In vitro and *in vivo* analysis of the functional significance of Tenascin-R and -C, CD24 and Semaphorin3A for neural stem cell behaviour and axonal pathfinding

in

Mus musculus (L.) 1758 and Rattus norvegicus (Berkenhout) 1769

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Abstract

Extracellular cues play crucial roles in the orderly development and plasticity of the nervous system, as well as in processes leading to disease and controlling regeneration.
A prominent group of the extracellular matrix represents the family of tenascins. Its members are involved in diverse functions such as cell migration, neurite growth, differentiation, axonal guidance and synaptic plasticity.
Neural stem cells are the precursor cells of the nervous system. Their multipotentiality and capability for self-renewal disclosed the prospect for a therapeutic use in the treatment of degenerative brain disorders.
The main aim of this work was to investigate the importance of the tenascins TNC and TNR for neural stem cell behaviour. By analyzing migratory activity, neurite growth and differentiation events of neural stem cells with the help of *in vitro* dissociated cell cultures and a newly established co-culture system of hippocampal organotypic slices with neural stem cells, evidence for an involvement of tenascins in neural stem cell development was found.
Whereas TNR deficiency or ectopic expression by neural stem cells did not affect their development, TNC deficiency led to increased relative numbers of astrocytes after differentiation was induced and to reduced percentages of cells that could be detected within deeper layers of the slice tissue they had been applied to. Unexpectedly by constitutively expressing TNC, cell migration could not be further enhanced but resulted in decreased numbers of mature NeuN positive neurons whereas the number of

young neuronally committed cells remained unchanged. Furthermore, indications for a modulation of the EGF receptor by TNC were found as its expression was prolonged or upregulated by differentiating stem cell cultures following increased TNC expression.
Thus, TNC may indeed be important in modulating neural stem cell development by playing a role in regulating astrocytic numbers and in coordinating neuronal maturation or survival and migration. Contradictory results under different experimental conditions as well as the modulation of the EGF receptor expression further suggest that TNC exerts its function by orchestrated multiple interactions.
Two further extracellular cues, Sema3A and CD24, both being implicated in neurite outgrowth and axonal guidance, were subject of a second study. Their expression at the sites of outgrowing corticospinal axons suggested a function in the correct pathfinding and timed outgrowth of the corticospinal tract. However, the examination of the tract in Sema3A and CD24 deficient mutant mice by *in vitro* and *in vivo* labelling methods using a fluorescent dye revealed no alterations of the timing and the pathfinding in the pons and at the level of the pyramidal decussation of corticospinal and corticopontine fibers.

Zusammenfassung

Für die korrekte Entwicklung des Nervensystems, seine Plastizität aber auch in degenerativen Prozessen oder bei Regeneration spielen extrazelluläre Signalmoleküle eine entscheidene Rolle. Eine grosse Gruppe von Molekülen der extrazellulären Matrix stellen die Tenascine dar. Ihre Vetreter sind an unterschiedlichen Funktionen beteiligt, u.a. an Zellmigration, Neuritenwachstum, Differenzierung, axonaler Wegfindung und synaptischer Plastizität.

Neurale Stammzellen sind die Vorläuferzellen des Nervensystems. Durch ihre Multipotentialität und die Fähigkeit sich selbst zu regenerieren eröffnete sich die Hoffnung auf eine therapeutische Anwendung in der Behandlung von neurodegenerativen Krankheiten.

Das Hauptziel dieser Arbeit war die Bedeutung der Tenascine TNC und TNR für das Verhalten von neuralen Stammzellen zu untersuchen. Durch Analyse von Migration, Neuritenwachstum und Differenzierung neuraler Stammzellen mit Hilfe von *in vitro* Kulturen von dissoziierten Zellen sowie einem neu-etablierten Co-Kultursystem, bestehend aus hippokampalen organotypischen Schnitten und neuralen Stammzellen, konnte eine Beteiligung von Tenascinen an der Entwicklung von neuralen Stammzellen gezeigt werden.

Während TNR -Defizienz oder -ektopische Expression keinen Einfluss auf neurale Stammzellen hatte, führte das Fehlen von TNC zu einem Anstieg der Zahl der Astrozyten nachdem die Zellen zur Differenzierung angeregt wurden, und zu einer

verringerten Zahl von Zellen, die innerhalb des Schnittegewebes detektiert werden konnten, auf das sie zuvor appliziert worden waren. Entgegen den Erwartungen konnte das Migrationsverhalten der Zellen durch eine konstitutive TNC Expression nicht weiter gesteigert werden und führte hingegen zu einer Reduktion ausgereifter NeuN positiver Neurone, während sich die Zahl junger unreifer Neurone nicht veränderte. Weiterhin konnten Hinweise auf eine Modulierung des EGF Rezeptors durch TNC gefunden werden, da erhöhte TNC Expression zu einer Verlängerung oder Steigerung dessen Expression führte.

Durch Regulation der Astrozytenzahl und Koordination von neuronaler Reifung oder Überleben mit Migration könnte TNC eine wichtige Rolle für neurale Stammzellentwicklung spielen. Widerspüchliche Ergebnisse, die unter verschiedenen experimentellen Bedingungen erlangt wurden, sowie die Modulation der Expression des EGF Rezeptors legen weiterhin nahe, dass TNC seine Funktion durch konzertierte vielfältige Interaktionen ausübt.

Zwei weitere extrazelluläre Signalmoleküle Sema3A und CD24, beide involviert in Neuritenwachstum und axonale Wegfindung, wurden in einer zweiten Studie untersucht.

Da Sema3A und CD24 dort exprimiert werden, wo corticospinale Axone auswachsen, wurde vermutet, dass beide Moleküle bei korrektem zeitlichen Auswachsen und der Wegfindung des corticospinalen Trakts eine Rolle spielen. Die Untersuchungen des Trakts von Sema3A und CD24 defizienten Mausmutanten im Bereich der Pons und der pyramidalen Kreuzung durch *in vitro* und *in vivo* Färbemethoden mit Hilfe eines fluoreszierenden Farbstoffs ergaben jedoch keine Veränderungen von corticopontinen oder corticospinalen Fasern, weder in Hinblick auf das zeitliche Auswachsen noch in Hinlick auf eine korrekte Wegfindung.

Abbreviations

AMP	Ampicillin
AMP^r	Ampicillin resistance
APP	Amyloid precursor protein
APS	Ammoniumperoxodisulfate
bFGF	Basic fibroblast growth factor
BHK	Baby hamster kidney cells
BME	Eagle's basal medium
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumine
CA	*Cornu ammonis*
CAM	Cell adhesion molecule
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CNTF	Ciliary neurotrophic factor
CNPase	Cyclic nucleotide phosphodiesterase
CNS	Central nervous system
CREB	cAMP response element binding protein
CST	Corticospinal tract
Ct	Threshold cycle
D	Dalton (g / mol)
dATP	2'-desoxyadenosinetriphosphate
dCTP	2'-desoxycytosinetriphosphate
dd	Double distilled
DEPC	diethylpyrocarbonate
DiI	1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate
dGTP	2'-desoxyguanosinetriphosphate
DMEM	Dulbecco's modified Eagle's medium

DMSO	dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	2'-desoxyribonucleotide-5'-triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiotreitol
E	Embryonic
ECL	enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	ethylendiamin-N,N,N',N'-tetraacetic acid
EGF	Epidermal growth factor
EGFL	EGF-like
EGFR	Epidermal growth factor receptor
ES	Embryonic stem
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FNIII	Fibronectin type III
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP+	Green fluorescence protein
GPI	Glycosylphosphatidylinisotol
h	Human, hour
HBSS	Hank´s balanced salt solution
HEPES	2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid
HRP	Horseradish peroxidase
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
Ig	Immunoglobulin
IZ	Intermediate zone
kb	Kilo basepairs
LB	Luria Bertani
LTP	Long-term potentiation
MAG	Myelin-associated glycoprotein

MAP	Mitogen-activated proteinase
MBP	Myelin-basic protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOPS	(4-(N-morpholino)-propan)-sulfonic acid
mRNA	Messenger ribonucleic acid
NCAM	Neural cell adhesion molecule
NSC	Neural stem cell
OD	Optic density
P	Postnatal
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Poly-ethylene glycol
PFA	Paraformaldehyde
pGp9.5	neuronal protein gene product
PLL	Poly-l-lysine
PNS	Peripheral nervous system
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature, Reverse transcription
SAP	Shrimp alkaline phosphatase
SEM	Standard error of the mean
Sema3A	Semaphorin3A
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
SVZ	Subventricular zone
TA	Tenascin assembly
TAE	Tris-acetate buffer
TBS	Tris-buffered saline
TEMED	N,N,N’,N’-Tetramethylethylendiamin

TN	Tenascin
TNC	Tenascin-C
TNR	Tenascin-R
TNR/C+/+	Tenascin-R/C wild-type
TNR/C-/-	Tenascin-R/C deficient
Tris	tris(-hydroxymethyl)-aminomethane
U	Unit
UV	Ultra-violett
VZ	Ventricular zone
w / v	Weight per volume

General Introduction

During development several steps of organisation follow each other and grip into one another to form the highly complex still plastic nervous system. In vertebrates a pool of neuroepithelial cells proliferates expanding the cell mass of the central nervous system and giving rise to the different cell lineages neurons, astrocytes and oligodendrocytes. The production of neurons and glial cells is precisely controlled in number and timing. Neurogenesis occurs first and is followed by generation of glial cells. Neuroblasts are born in the ventricular and later on subventricular zone surrounding the ventricles, become postmitotic and start to migrate into their appropriate regions. On their journey they undergo a transformation into specifically differentiated neurons (expressing a characteristic set of receptor and transmitter systems) and start to extend axons into their synaptic targeting area. How is the differentiation of different cell types regulated? Is it external cues or internal programs that control the cell fate? How do cells and axons find their way into the appropriate region? Up to now the ultimate answers to these questions are lacking. Known is that these developmental processes depend on interactions between cells and their surrounding matrix and among the cells themselves. A pattern of external cues is thought to induce the activation of characteristic transcription factors early in development that by activating subsequent cascades of proteins lead to the development of different cell types. Migrating cells and outgrowing processes orient themselves along short range and long range, attractive or repulsive

guidance cues. These guidance cues can be expressed on the cell surface of for instance glial cells or pioneering axons or they are secreted into the extracellular matrix.
In this work we examined the properties of the extracellular present proteins tenascin-C (TNC), tenascin-R (TNR), semaphorin3A (Sema3A) and CD24 to influence developmental events such as differentiation, migration, neurite growth and axonal pathfinding.
The first study will focus on neural stem cells and in which way tenascins (TNs) influence their differentiation and behaviour.
Guidance of axonal growth cones is the topic of the second study which investigates pathfinding errors of the corticospinal tract in Sema3A and CD24 deficient mutant mice.

Study one: Tenascin functions in neural stem cell behaviour

1.0. Introduction

1.1. Stem cells and their definitions

The nature of stem cell function dictates the complement of specific functional attributes that stem cells must be endowed with. In the absence of any identifying antigenic markers, these functional attributes provide the only basis for a reliable identification of stem cells. The most widely accepted definition identifies stem cells as: a) undifferentiated cells, that lack markers of differentiated tissue specific cells, b) capable of proliferation and, more importantly, c) possessing self-renewal capacity, d) able to generate functionally differentiated progeny and e) able to regenerate the tissue after injury (Löffler et al., 1997). Some terms are given greater weight in identifying a candidate stem cell: either self-renewal or the capacity to generate a wide array of differentiated progeny, or the ability to regenerate a tissue may be accepted, even alone to identify a stem cell (Morrison et al., 1997). The proof for stem cell identity relies on *in vitro* demonstrating that different phenotypic cells can be generated from of a single cell and that this multipotentiality is maintained by the cells over time undergoing several subcloning steps.

1.2. Stem cells in the development of the nervous system

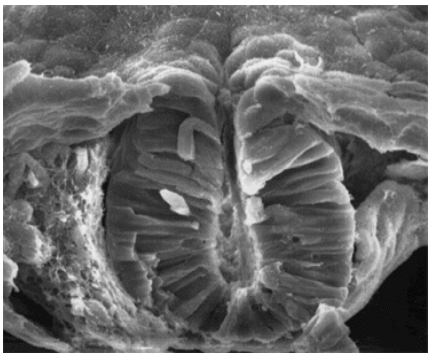


Fig. 1 Neural tube
Electron raster microscopic picture of a mouse neural tube at E8. The transverse section shows the long columnar neuroepithelial cells lining the lumen of the tube.

With the closure of the neural tube in the early vertebrate development the neural crest cells start migrating and give rise, among others, to the cells of the peripheral nervous system. At this stage (at embryonic day 8 (E8) in mouse) the lumen of the neural tube is lined with a ventricular layer of primitive neuroepithelial cells. They have a columnar appearance, touching ventricle and pial surface during the cell cycle (Fig. 1). These cells proliferate and produce most of the cell types of the future central nervous system. The majority of these cells inherits a pluripotent potential with the ability to give rise to neuronal and glial lineages and to regenerate themselves and therefore can be described as neural stem cells (NSCs) (Kalyani et al., 1997). In a first wave of proliferation, the neurogenic phase from E12 to E20 in rodents, the ventricular layer within the spinal cord produces neuroblasts (future neurons) that migrate peripherally to form an outer mantle zone, the future grey matter of spinal cord and brainstem. The nerve fibres from the neuroblasts form a marginal zone, superficial to the mantle zone, which contains fibre tracts (future white matter of the spinal cord and brainstem). The second wave of proliferation, the gliogenic phase starts around birth and produces glioblasts that migrate peripherally and become astrocytes and oligodendrocytes (Das, 1977). In the cerebral cortex the neuroepithelial cells lining the ventricles form the ventricular zone. Organized migration processes lead to the typically six-layered cortex. Hereby the first postmitotic cells migrating in a radial fashion out of the ventricular zone are forming the later on innermost layers. Latterly born neurons bypass earlier-generated neurons to form the cortical layers in an inside-out sequence (Brown et al., 1991). Throughout embryonic development radial glia cells continue to contact both ventricle and pia, guiding neuronal migration and in addition as recent data suggest also producing cortical neurons and glia (Tamamaki et al., 2001).

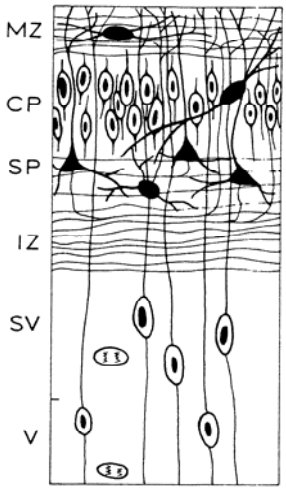


Fig. 2 Schematic drawing of the cortical lamination in mouse at E14
MZ: marginal zone; CP: cortical plate; SP: subplate; IZ: intermediate zone; SV: subventricular zone; V: ventricular zone
From Uylings et al., 1994.

At mid-gestation around E13 in mouse, a second layer of proliferating cells appears between the ventricular zone and the adjacent intermediate zone, the subependymal or subventricular zone (Fig. 2). This germinal zone contains cells, produced in the ventricular zone, which after the neurogenic period ceased, mainly give rise to glia. The subventricular zone expands greatly in late gestation and in early postnatal life. By postnatal ages the radial glia have transformed into astrocytes and the ventricular zone disappears but the subventricular zone remains into adulthood in some areas harbouring stem cells that still have the potential to give rise to glia and neurons (Super et al., 1998).

1.2.1. Embryonic stem cells

Embryonic stem cells (ES cells) are derived from the inner cell mass of developing blastocysts (Martin, 1981). Murine ES cells are demonstrated to be pluripotent, because in addition to their self-renewal capacity they are able to differentiate into cellular derivates of all three primary germ layers.

Neural stem cells (NSCs) are neural precursor cells that are able to self-renew extensively and can be propagated for months, displaying a steady capacity to generate neurons, astrocytes and oligodendrocytes. Originally NSCs were thought to have a limited potential for the production of differentiated derivates and were classified as multipotential rather than pluripotential but recent studies have questioned that view. These studies show that neural stem cells e.g. can form blood cells and muscle tissue (Bjornson et al., 1999). Embryonic NSCs have been isolated from different CNS regions, i.e. striatum, cortex, spinal cord, thalamus, ventral mesencephalon of both human (Vescovi et al., 1999; Quinn et al., 1999) and rodent embryos (Reynolds and Weiss, 1992; Davis and Temple, 1994; Kilpatrick and Bartlett, 1995; Qian et al., 1997) at different stages of development.

1.2.2. Adult stem cells

In the adult organism stem cells play an essential homeostatic role by replacing differentiated tissue cells 'worn out' by physiological turnover or lost to injury or disease. In the adult organism stem cells are still present in various tissues such as testis, mammalian skin, gut or bone marrow (Spradling et al., 2001). De novo neurogenesis has been shown to occur in two discrete areas of the adult CNS of mammals, namely the subventricular zone and the dentate gyrus of the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Corotto et al., 1993; Luskin, 1993). The neural precursor cells in the subventricular zone represent a supply of interneurons for the olfactory bulb (Altman, 1969; Corotto et al., 1993; Luskin, 1993; Lois and Alvarez-Buylla, 1994). In the adult hippocampus neural precursor cells are generated in the subgranular zone of the dentate gyrus and differentiate into neuronal and glial cells in the granular layer of the dentate gyrus (Cameron et al., 1993) and the cortex (Kaplan, 1981; Huang and Lim, 1990; Gould et al., 1999). The fact that *in vivo* newly generated neurons could be labelled did not yet mean that the precursor cells were stem cells as evidence for multipotentiality and / or self-renewal capacity was lacking. Such could subsequently be validated for cells isolated from striatum, subventricular zone, hippocampus, olfactory bulb and cortex of adult rodent and human, and spinal cord of adult mouse and rat tissue (Reynolds and Weiss, 1992; Weiss et al., 1996; Gritti et al., 1996, 1999; Johansson et al., 1999; Taupin and Gage, 2002).

1.3. Importance in gene therapy

The multipotentiality of stem cells and the possibility to extensively expand their number in culture raised hopes to use stem cells to treat degenerative diseases in humans such as Parkinson's disease, Huntington's disease, multiple sclerosis or spinal cord injury (Drucker-Colin and Verdugo-Diaz, 2004). During recent years various studies have investigated this area with some remarkable evidence (Temple, 2001). ES cells transplanted into a Parkinson rat model e.g. developed into functional dopaminergic neurons (the neuronal cell type whose loss characterizes the disease) and improved motor functions of the lesioned animals (Björklund et al., 2002). The advantages of ES cells for therapy are pluriopotency and a high migratory potential,

however, a major hurdle is the frequently observed formation of teratocarcinomas following ES cell transplantations; in some animals ectopic mesodermal and epithelial cells were found in nervous tissue (Björklund et al., 2002). One pursued path to overcome this is the transplantation of *in vitro* predifferentiated ES cells. Completely predifferentiated cells exhibit the lowest risk but they show a reduced survival rate and loose some advantageous characteristic such as migratory capacity (Murray et al., 2002) that could be crucial for therapy of diseases that show a broader degeneration e.g. multiple sclerosis. An alternative migrating and pluripotent cell type for therapy represents the NSC that is described not to form tumours (Winkler et al., 1998; Englund et al., 2002).

Transplantation studies stressed the importance of extracellular cues for NSC development: Migration of transplanted cells is reduced in adult non-neurogenic brain regions compared to embryonic brain (Winkler et al. 1998) whereas in neurogenic adult brain regions transplanted cells still exhibited a pronounced migratory potential (Fricker et al., 1999). On the one hand NSCs acquire positional and temporal information that specifies and restricts their differentiation. E.g. mid/hindbrain progenitor cells lose their ability to generate telencephalic phenotypes after E13.5 in mouse (Olsson et al., 1997; Temple, 2001). On the other hand some of this inherent information can be reversed (Hack et al., 2004) such that multipotent embryonic and adult progenitor cells generate region-specific heterotypic cell types *in vivo* when exposed to the appropriate environmental cues. E.g. *in vitro* propagated human striatal foetal NSCs transplanted into the hippocampus differentiated into neurons characteristic for the layers they incorporated into (Fricker et al., 1999). The important impact of the host tissue is further stressed by transplantation studies using animal models of brain injury or diseases. Specific cell loss in mouse models seems to enhance the generation of the desired formerly degenerated cell type by suggestively changed environmental cues (Snyder et al., 1997; Wennersten et al., 2004; Pluchino et al., 2003; Richardson et al., 2005). E.g. neuronal differentiation of neural precursors in the adult mouse brain was observed in particular within areas of apoptotic cell death (Snyder et al., 1997). Whereas human NSCs develop after transplantation into a rat model of traumatic brain injury into neurons and astrocytes but not oligodendrocytes (Wennersten et al., 2004) in a chronic model of multiple sclerosis the majority of transplanted adult mouse NSCs

differentiated into oligodendrocytes, suggesting influences of inflammatory events and molecules that are expressed by the host tissue (Pluchino et al., 2003).

The fate and behaviour of stem cells evidently depends on environmental cues and cell intrinsic properties that probably interact with each other. Which are the environmental factors that influence the migration and differentiation of endogen and transplanted stem cells and which mechanisms are employed? To unravel such questions will give insight into the development of the central nervous system and will be an important prerequisite in order to control and direct stem cell fate into the desired direction and enable a risk less employment of stem cells in therapy. Understanding which cellular characteristics make a NSC and which events guide them to different neural cell fates could also open up strategies in the third thought therapeutic use of stem cells which is engaging the endogen pool of NSCs in the CNS.

Several factors of the extracellular milieu have been demonstrated to have important functions in maintaining the multipotentiality of stem cells, regulating proliferation and correctly timed differentiation as well as migration of progenitor cells and neurite growth (Johe et al., 1996; Cameron et al., 1998; Marin and Rubenstein, 2003; Davies, 2000). E.g. the platelet-derived growth factor (PDGF) was reported to induce neuronal differentiation of neural stem cells and the ciliary neurotrophic factor (CNTF) instructively induces astrocytic differentiation (Johe et al., 1996). The basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) act as mitogens for neural stem cells and are implicated in differentiation events (Johe et al., 1996; Gritti et al., 1996; Reynolds and Weiss, 1996; Lilien, 1995). The EGF receptor, a tyrosine kinase receptor, additionally plays a role in migration such as radial movement in the telencephalon (Kornblum et al. 1997; Burrows et al.; 1997; Caric et al., 2001). Various other molecules have been described to take part in stem cell modulation such as neurotrophins, neurotransmitters, cell adhesion molecules, Sonic hedgehog (Shh) or the wingless-type (Wnt) genes (Echelard et al., 1993; McMahon and Bradley, 1990; Cameron, 1998; Dihne et al., 2004).
A prominent family of proteins present in the extracellular matrix (ECM) during the development of the nervous system are tenascins (TNs). In our first study we were

interested by which means TNs play a role in controlling or influencing NSC fate and behaviour. An introduction into the family of TNs is given below.

1.4. The TN family of ECM glycoproteins

TNs are a growing family of glycoproteins present in the ECM in many tissues throughout the body (Jones and Jones, 2000; Chiquet-Ehrismann, 2004). At present five TN genes are identified in the vertebrate genome, termed TNC, TNR, TNX, TNW and TNN (Bristow et al., 1993; Chiquet-Ehrismann et al. 1994; Erickson, 1994; Hagios et al., 1996; Weber et al., 1998, Neidhart et al., 2003).

The first member of the TN family, TNC, was discovered independently by several laboratories investigating different aspects of cell, developmental or tumour biology. Accordingly, it has been given a number of names including glioma mesenchymal extracellular matrix antigen (GMEM) (Bourdon et al., 1983), myotendinous antigen (Chiquet and Fambrough, 1984), hexabrachion (Erickson and Inglesias, 1984), J1 200/220 (Kruse et al., 1985), cytotactin (Grumet et al., 1985) or neuronectin (Rettig et al., 1989). The name TN represents a combination of two Latin verbs, tenere (to hold) and nasci (to be born) (Chiquet-Ehrismann et al., 1986). TNC is highly conserved during evolution and most prominently expressed during development, not only in the nervous systems but also in several non-neuronal tissues. For instance, TNC is expressed in tendons and bones (Chiquet and Fambrough, 1984; Mackie et al., 1987), kidney and gut (Aufderheide et al., 1987; Aufderheide and Ekblom, 1988), mammary glands (Chiquet-Ehrismann et al., 1986), or teeth (Thesleff et al., 1987). Moreover, TNC becomes re-expressed upon oncogenesis (Chiquet-Ehrismann et al., 1986; Vollmer, 1994), following adult brain injury (Laywell et al., 1992), and at the margins of healing wounds (Mackie et al., 1988).

Expression of TNR, also known as restrictin, janusin, or J1 160/180 (Pesheva et al., 1989; Fuss et al., 1991; Rathjen et al., 1992; Norenberg et al., 1992), is restricted almost exclusively to the CNS, in a pattern partially overlapping with that of TNC. TNR is found in retina, cerebellum, and spinal cord of embryonic chicken (Norenberg et al., 1992), and in retina, optic nerve, cerebellum, hippocampus, and olfactory bulb of

postnatal mice with a peak of expression between P8 and P15 (Wintergerst et al., 1993). Transient expression of TNR, however, is also detectable in the PNS (Probstmeier et al., 2001).

Other members of the TN family show partially overlapping expression patterns with TNR and TNC. E.g. TNW that was identified in the zebrafish is expressed prominently in neural crest pathways and colocalizes with TNC in several tissues during development TNW. However, it is absent in the CNS and in the axial mesoderm, both being TNC positive structures (Weber et al., 1998). In the avian nervous system, there is some overlap in the expression patterns of TNY with that of TNC during development and between TNY and TNR in adulthood (Tucker et al., 1999) and TNN expression is detectable in all brain regions, with a characteristic staining pattern in the TNR and TNC positive hippocampus (Neidhardt et al., 2003).

1.4.1. Common structure of TNs

All TNs share a common consecutive arrangement of protein domains (Fig. 3). The protein modules making up TNs include heptad repeats, EGF-like repeats (EGFL), fibronectin typeIII (FNIII) repeats, and a C-terminal globular domain shared with fibrinogens. These protein domains are lined up and give rise to long and extended molecules. At the N-terminus TNC,-R and -W have a TN assembly (TA) domain, which contains cysteine residues and three to four alpha helical heptad repeats (Conway and Parry, 1991) that enable the linkage into oligomeric structures. In case of TNC and TNW oligomerization leads to the formation of hexamers or hexabrachion as visualized by rotary shadowing and electron microscopy while TNR has been isolated as a trimeric molecule (Erickson, 1994; Vaughan et al., 1987; Husmann et al., 1992). The heptad repeats are also contained in TNX and TNY, which also assemble into oligomers. The amino terminal domain is followed by EGFL repeats and a region consisting of FNIII domains. The EGFL repeats are 31 amino acids in length and contain six cysteine residues that participate in intrachain disulfide bonds. FNIII domains contain approximately 90 amino acids and are extended globular structures composed of seven anti-parallel beta-strands arranged in two sheets (Patthy, 1990). The number of these repeated domains varies between the family members. Structurally TNY differs more

from the mammalian TNX than other orthologous TNs differ between each other. Such that TNY contains a single EGFL repeat while TNX has eighteen of them. TN EGFL domains form an exceptionally compact structure compared to that of the epidermal growth factor (Cooke et al., 1987), and lack the acidic residues required for binding of calcium (Jones and Jones, 2000). The number of FNIII domains in TNC –R, -X and –N are altered by alternative splicing, generating a great diversity of polypeptides that partly show distinct expression. Assembly of heterotypic TN multimers might provide additional combinatorial diversity of ECM structure and function in cellular contexts in which more than one TN gene or splice variant is present (Jones and Jones, 2000; Neidhardt et al., 2003). The terminal knob of the TNs is composed of a globular domain that resembles the carboxy terminal portion of the beta and gamma chains of fibrinogen. TNs carry numerous N- and O-linked sugar chains for instance the HNK-1 carbohydrate, sialylated glycans and chondroitin sulfate glycosaminoglycans (GAGs) that are spatially and temporally regulated and contribute to the functional role of TNs (Woodworth et al., 2002; Pesheva et al., 1989; Probstmeier et al., 2000; Fuss et al., 1993; Saghatelyan et al., 2004).

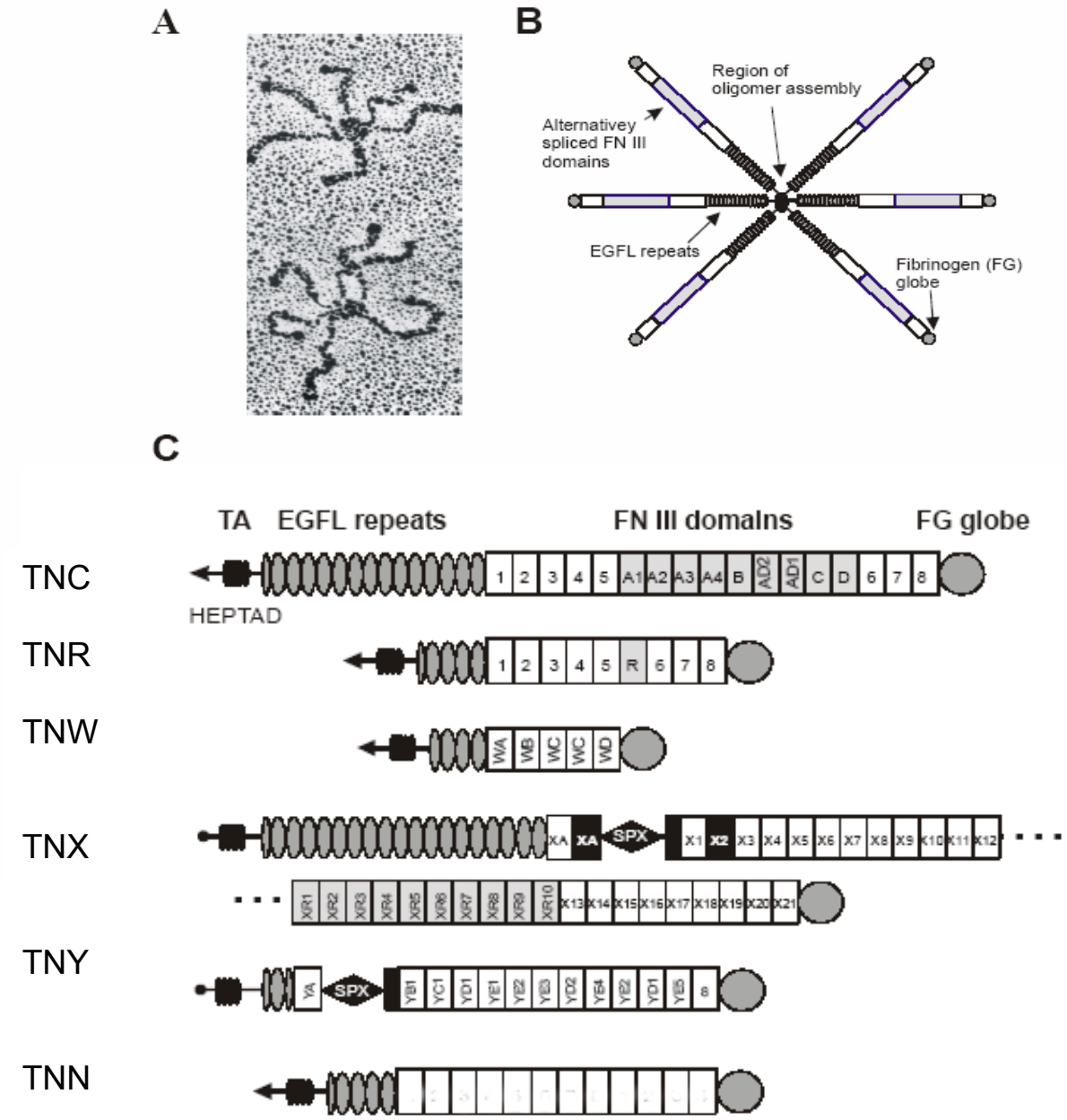


Fig. 3 Structure of the TNs**. A**: Rotary shadowing image of two mouse TNC hexabrachions. Each arm is approximately 100nm in length. **B**: Model of the TNC hexabrachion. The TN assembly (TA) domain links six TNC chains via the heptad repeats (see C). **C**: Schematic diagrams of described TNs. Proceeding from left to right, the domains are as follows: TA domain, an array of EGFL repeats (ovals), two types of FNIII domains: those conserved in all variants of TNC (white rectangles) and those that are alternatively spliced (grey rectangles), and the terminal fibrinogen globe (circle). The figure has been adopted from Jones and Jones (2000) and modified with addition of TNN.

1.4.2. The extracellular matrix glycoprotein TNC

As the first identified member of the TN family of the four TNs that are detectable in the nervous system (TNC, TNR, TNX and TNN) TNC has been most extensively studied (Faissner, 1993; Bartsch, 1996; Jones and Jones, 2000; Joester and Faissner, 1999).

In mouse TNC the N-terminal TA domain is followed by 14.5 EGFL repeats and eight constitutively expressed FNIII modules. As already mentioned the nature and number of FNIII domains are altered by alternative RNA splicing depending on the species, regions and developmental stages investigated. Up to nine alternatively spliced FNIII domains are positioned between the constitutively expressed FNIII repeats five and six (Jones et al., 1989; Gulcher et al., 1991; Saga et al., 1991; Siri et al., 1991; Weller et al., 1991; Sriramarao and Bourdon, 1993; Doerries and Schachner, 1994; Meiners and Geller, 1997). 27 different FNIII variants of murine TNC have been documented, indicating that many of the theoretically possible combinations of FNIII repeats are likely to be expressed during tissue morphogenesis (Joester and Faissner, 1999; Joester and Faissner, 2001). Additionally, FNIII repeats are also susceptible to proteolytic degradation, allowing TNC-containing matrices to be selectively remodelled, particularly by matrix metalloproteinases (MMPs) and serine proteases (Imai et al., 1994; Siri et al., 1991; Gundersen et al., 1997). Biochemically TNC glycoproteins display apparent molecular weights ranging from 190 kD to 240 kD (Bartsch et al., 1992).

1.4.3. Expression pattern of TNC in the nervous system

In the nervous system of mouse TNC is first detected at E10 (Kawano et al., 1995) and expressed in a regionally and temporally developmentally regulated pattern. For example in newborn mice TNC is abundantly expressed in the cerebellar cortex and is down-regulated with increasing age, reaching adult levels at the end of the first postnatal month (Bartsch et al., 1992). Interestingly, the expression of different isoforms is differentially regulated: the high molecular weight isoforms of 225 kD and 240 kD are more drastically down-regulated than the low molecular weight isoforms of 190 kD and 200 kD (Bartsch et al., 1992). TNC is mainly expressed by immature and reactive

astrocytes. For instance, TNC is secreted by subsets of radial glial cells, and Bergmann glial cells during neuronal differentiation and neuronal migration in the cerebral and cerebellar cortex, respectively (Crossin et al., 1986; Kawano et al., 1995; Bartsch, 1996; Yuasa, 1996). Expression of TNC by subclasses of neurons has also been demonstrated, among them, immature neurons such as granule cells in the hippocampus, and motoneurons of the spinal cord as well as horizontal cells in the developing and adult mouse retina (Kawano et al., 1995; Zhang et al., 1995; Ferhat et al. 1996, Bartsch, 1996). In the adult brain, TNC persists in areas, many of them known to retain a high degree of plasticity. These brain regions include certain nuclei of the hypothalamus (Theodosis et al., 1997), the olfactory system (Gonzalez and Silver, 1994), the cerebellar cortex, the retina, the proximal unmyelinated part of the optic nerve (Bartsch, 1996), and the hippocampus (Ferhat et al., 1996; Nakic et al., 1998). A developmental regulation of TNC expression levels has also been observed in the PNS. Intense and widespread TNC immunoreactivity is detectable in developing peripheral nerves, whereas the protein is restricted to the perineurium and to nodes of Ranvier in adult nerves (Martini, 1994; Bartsch, 1996).

1.4.4. Functional characterization of TNC

High levels of TNC expression at critical stages of neuronal development, regeneration and synaptic plasticity in the adult, have prompted several laboratories to investigate functional properties of this ECM constituent *in vitro*. These studies have implicated that diverse functional domains, including those mediating cell proliferation, migration, axon guidance and tissue development and repair, coexist in the native molecule. Experiments with neuronal cells have revealed that the protein exhibits functional dichotomy.

For example, TNC has been demonstrated in numerous studies to be adhesive or anti-adhesive (Grumet et al., 1985; Kruse et al., 1985; Tan et al., 1987; Faissner and Kruse, 1990; Doerries et al., 1996), to support or restrict cell migration of certain cell types (Tan et al., 1987; Husmann et al., 1992), and to promote or inhibit neurite outgrowth (Faissner and Kruse, 1990; Lochter et al., 1991; Lochter and Schachner, 1993; Bartsch, 1996; Doerries et al., 1996). Examples for different functional implications are given below.

Adhesion

Originally, TNC has been described to mediate adhesion between CNS glial cells and neurons (Grumet et al., 1985; Kruse et al., 1985). Subsequently it became apparent that TNC is anti-adhesive rather than adhesive for the attachment of a variety of cell types of the CNS and PNS e.g. neural crest cells (Bartsch, 1996). Experiments using monoclonal antibodies or recombinantly expressed TNC fragments could demonstrate that distinct sites within the TNC molecule exhibit either adhesive or anti-adhesive effects. The differential cellular response may depend on specific expression of distinct cellular receptors or alternatively on differentially linked intracellular signalling (Bartsch, 1996).

Migration

The presence of TNC at sites of active cell migration (Tan et al., 1987; Tucker and McKay, 1991; Bartsch et al., 1992; Bartsch et al., 1995) suggested a role for TNC in cell motility or guidance of migrating cells. TNC is strongly expressed during the period of cerebellar granule cell migration from the external to the internal granular cell layer and antibodies against TNC interfere with this process (Husmann et al., 1992).

A role for TNC in regulating oligodendrocyte migration was suggested due to its expression and was validated *in vitro* (Bartsch et al., 1992; Bartsch et al., 1994 Frost et al., 1996; Kiernan et al., 1996) and *in vivo* as in TNC deficient mice the rate of oligodendrocyte precursor migration along the optic nerve is increased. Additionally, TNC is expressed at high levels in migratory tumour cells and was shown to support migration of glioma cells in culture. Thus it may contribute to the high invasive potential of these tumour cells (Kleihues et al., 1995, Phillips et al., 1998). In addition TNC was found to both inhibit and promote migration in the PNS: E.g. Schwann cell precursors do round up and do not move on TNC substrate (Wehrle-Haller and Chiquet, 1993). In contrast neural crest cell migration is enhanced *in vitro* on TNC substrate and inhibited by down-regulation of TNC protein (Halfter et al., 1989; Tucker, 2001).

Proliferation

In addition to the accelerated migration of oligodendrocyte progenitors in the TNC deficient mouse (see above) reduced rates of proliferation in different regions of the CNS, such as the cortex, corpus callosum and striatum are described. A paracrine/autocrine regulation of astrocytic proliferation was suggested upon the finding that proliferation of activated cultured astrocytes is reduced with application of a function blocking anti-TNC antibody (Nishio et al., 2003).

Differentiation

Oligodendrocyte precursor cells have been found to mature accelerated in the absence of TNC with earlier morphological differentiation and precocious expression of myelin basic protein (Garwood et al., 2004). A recent study involves TNC in stem cell differentiation: Mice lacking TNC show a delayed acquisition of the EGFR in the subventricular zone and altered numbers of NSCs which give rise to neurons with an increased probability (Garcion et al., 2004).

Neurite elongation

In the developing CNS, expression of TNC has been consistently observed in regions of active axonal growth. For instance, thalamocortical afferents, retinal ganglion cell axons, or parallel fibers in the cerebellar cortex elongate in a TNC-rich environment (Bartsch, 1996). In the chick retino-tectal system, TNC is associated with the cell surface of glial fibers and growing axons located in the vicinity of glial processes at times when retinal ganglion cell axons arrive (Bartsch et al., 1995) which suggests that TNC may not only support axonal growth but in addition may guide axons to their appropriate targets *in vivo*. However, experimental evidence *in vitro* is contradictory and strongly depends on how the molecule is offered and which cell type is looked at. Several studies support a role for TNC as a guiding, growth restricting molecule as it inhibits neurite growth or growth cone advance in particular if presented as a border of (Faissner and Kruse, 1990; Crossin et al., 1990; Taylor et al., 1993; Krull et al., 1994). The *in vivo* counterpart to *in vitro* examined boundaries is a TNC accumulation in anatomically well-defined boundaries in the brain. TNC transiently delineates barrels in the developing somatosensory cortex of rodents, several brain nuclei, distinct axon

fascicles and olfactory glomeruli (Crossin et al., 1989; Steindler et al., 1989; Bartsch et al., 1992; Bartsch et al., 1994; Mitrovic et al., 1994; Krull et al., 1994; Gonzalez and Silver, 1994).
Importantly, evidence for growth promoting or attractive functions of TNC exists (Wehrle and Chiquet, 1990; Lochter et al., 1991; Hussmann et al., 1992; Taylor et al., 1993; Bartsch et al., 1995; Götz et al., 1996; Meiners and Geller, 1997; Meiners et al., 1999). Meiners et al. could show that neurites prefer to grow onto TNC substrate even if intermingled with otherwise repellent molecules and found this function to depend on the alternative spliced region (Meiners et al., 1999).

Synaptic plasticity

TNC expression correlates with synapse formation (D'alessandri et al., 1995), is coexpressed with TNR during formation of perineuronal nets (Hagihara et al., 1999) and is up-regulated activity dependent (Nakic et al., 1996; Nakic et al., 1998). Perturbation experiments and studies on TNC deficient mice stress the importance of TNC for the formation of neuromuscular junctions (Cifuentes-Diaz et al., 1998; Mege et al., 1992; Langenfeld-Oster et al., 1994), synaptogenesis and the plasticity of neuronal connections. E.g. the TNC deficient mouse exhibit reduced LTP in the CA1 region of the hippocampus which is suggestively due to a reduction in L-type voltage-dependent Ca^{2+}-channel signalling (Evers et al. 2002).

Further morphological changes in the cortex of a TNC deficient mouse support a role for TNC in developmental events, including increased neuronal density, astrogliosis and low density of parvalbumin-positive interneurons. In addition the ratios of oligodendrocytes to neurons and of inhibitory to excitatory neurons in the TNC deficient animals are reduced as opposed to the non-deficient animals (Irintchev et al., 2005). However which cellular events underlie these changes is not known yet.

Behaviour

TNC deficiency leads to behavioural alterations in mutant mice such as hyperactivity and abnormal swimming, in fact drowning (Fukamauchi et al., 1996) and slightly detectable hyperlocomotion (Kiernan et al., 1999).

In the lesioned nervous systems, expression of TNC has been reported to be up-regulated in a variety of lesion paradigms. E.g., stab wounds induce an up-regulation of TNC by Golgi epithelial cells in the cerebellar cortex (Bartsch, 1996) and by reactive astrocytes in the cerebral cortex (Laywell et al., 1992) suggesting that TNC with its inhibitory properties on neurite elongation might play a role in the non-permissive environment for neurite growth that is one of several reasons for the lack of substantial axonal regeneration in the adult CNS (Qiu et al., 2000). However, the finding that axons grew into the highly TNC immunoreactive lesion site following spinal cord injury questioned this hypothesis (Zhang et al., 1997). In addition it has to be noted that unaltered or decreased expression of TNC has also been observed in diverse lesion paradigms (Bartsch, 1996).

Opposing activities that are localized to distinct domains of TNC seem to be responsible for conferring adhesion, anti-adhesion, neurite outgrowth promotion, attraction, migration or repulsion of neurites or cell bodies and presumably arise as a consequence of interactions of TNC with other components of the ECM and cell surface receptors which are linked to a variety of intracellular signal transduction pathways (Götz et al., 1996; Kiernan et al., 1996; Meiners et al., 1999; Meiners et al., 2001).

1.4.5. TNC interactions

The number of putative TNC receptors is still increasing (Table 1). However, a correlation between binding sites of certain interaction molecules with effects observed *in vitro*, not to mention *in vivo*, has proven to be challenging.

	Interaction partner	**Reference**
Cell surface receptors:	CAMs including contactin / F3 and TAG-1 / axonin	Rigato et al., 2002; Milev et al., 1996
	Integrins	Mercado et al., 2004; Varnum-Finney et al., 1995; Garcion et al., 2001
	N-syndecan	Salmivirta et al., 1991
	CALEB	Schumacher and Stube, 2003
	Phosphacan	Jones and Jones, 2000
	AnnexinII	Chung et al., 1996
	Voltage-dependent sodium channels	Srinivasan et al., 1998
	Epidermal growth factor receptor (EGFR)	Swindle et al., 2001
ECM interactions:	Aggrecan, versican, brevican, neurocan, fibronectin	Jones and Jones, 2000

Table 1 Summary of TNC interactions

1.4.6. The extracellular matrixmolecule TN-R

TNR shows a strong homogeneity to TNC and an overall identity of 47 % in mouse to TNC. Thus the structure is highly similar: The cysteine-rich N-terminal region is followed by 4.5 EGFL domains, 8 FNIII like repeats and the fibronogen homologous C-terminus. Two isoforms are generated by alternative splicing of the sixth FNIII domain

(Jones and Jones, 2000). TNR glycoproteins are expressed in the CNS of different vertebrates as two major molecular forms of 160 kD and 180 kD (Pesheva et al., 1989).

1.4.7. Expression pattern of TNR

Also TNR expression is temporally regulated. During brain development TNR appears perinatally when the 180 kD form is first detectable. The protein has been found to be expressed by oligodendrocyte progenitors and type-2 astrocytes. It is abundant during the phase of active myelination and within the pathways of oligo precursor migration. After myelination has ceased TNR levels are down-regulated to lower adult levels (Bartsch et al., 1993; Wintergerst et al., 1993). In the adult it is mainly associated with and generated by oligodendrocytes. It is detectable at contact sites between unmyelinated axons, between myelin sheets and is highly accumulated at the nodes of Ranvier (Bartsch et al., 1993). TNR is also expressed by small subsets of CNS neurons, such as interneurons and motoneurons in spinal cord, retina, cerebellum and hippocampus (Fuss et al., 1993; Wintergerst et al., 1993; Weber et al., 1999). It is localized at perineuronal nets (often in association with TNC) that surround inhibitory interneurons (Celio and Chiquet-Ehrismann, 1993; Wintergerst et al., 1996). Interestingly the expression of neurons is not down-regulated in adulthood (Fuss et al., 1993). In addition TNR protein is localized in distinct zones of the olfactory bulb (Saghatelyan et al., 2004).

1.4.8. Functional characterization of TNR

As described for TNC also TNR exhibits dichotomy in respect to adhesive, migratory or neurite outgrowth modulating functions.

Adhesion

TNR has been demonstrated *in vitro* to be anti-adhesive for cerebellar neurons (Pesheva et al., 1989) and microglia but adhesive for chicken retinal neurons (Norenberg et al., 1995) and hippocampal neurons (Lochter et al., 1994), astrocytes and oligodendrocytes (Pesheva et al., 1989, 1997).

Neurite outgrowth

As a homogenous substrate TNR enhances axon growth of e.g. dorsal root ganglion cells (Taylor et al., 1993) while growth of neurites of other cell types in inhibited (Taylor et al., 1993; Pesheva et al., 1991). Interestingly just as TNC, TNR enhances neuronal polarity (Pesheva et al., 1994; Lochter et al., 1993). If presented as a sharp border TNR repels neurites of single neurons or explants *in vitro* (Pesheva et al, 1991, 1994; Taylor et al., 1993; Becker et al., 2000).

Migration

The expression of TNR along the pathways of oligodendrocyte progenitor migration points to a role for TNR function. In this context the glycoconjugates present on the TNR core protein are of functional importance. TNR linked chondroitin sulfate GAGs promote oligodendrocyte migration from white matter explants and increase the motility of oligodendrocyte lineage cells (Probstmeier et al., 2000). In addition a role for TNR in initiating certain aspects of migration of neuroblasts in the olfactory bulb was demonstrated (Saghatelyan et al., 2004).

Differentiation

An important finding is that TNR stimulates maturation of oligodendrocyte precursors, by a suggested autocrine mechanism (Pesheva et al., 1997). It has been proposed that timed differentiation of oligodendrocytes is due to an accumulation of TNR to a critical threshold triggering the differentiation program (Pesheva and Probstmeier, 2000). However *in vivo* relevance of this hypothesis still needs to be demonstrated as formation of myelin appeared normal in TNR deficient mice (Weber et al., 1999).

Synaptic plasticity

The expression of TNR in perineuronal nets that possibly function in synaptic stabilization indicated a role for TNR in formation, maintenance of such nets or in some forms of synaptic remodelling. TNR deficiency in a mutant mouse leads to a structural disruption of the ECM components (Weber et al., 1999; Bruckner et al., 2000). Antibodies against the HNK-1 epitope carried by TNR and the lack of TNR in mutant mice both lead to reduced perisomatic inhibition in the CA1 region of the hippocampus which may be a

consequence of observed structural alterations in perisomatic inhibitory synapses (Saghatelyan et al., 2000, 2001; Nikonenko et al., 2003).

Behavioural analysis of TNR deficient mice revealed various alterations including comprised motor coordination, increased anxiety and deficits in associative learning (Freitag et al., 2003; Montag-Sallaz and Montag, 2003).

In the lesioned nervous system, TNR expression is altered in a variety of paradigms. After peripheral nerve injury TNR becomes down-regulated in the corresponding motor nuclei. On the basis of the findings that activated microglia migrate into the lesioned nucleus and TNR exerts anti-adhesive and anti-migratory properties for microglia *in vitro* a role of TNR in neuronal protection was suggested (Angelov et al., 1998; Liao et al., 2005).
TNR has been shown to be up-regulated at the lesion site after transection of the postcommissural fornix in adult rat (Probstmeier et al., 2000) and in the hippocampus after induced epileptic seizures (Brenneke et al., 2004). Particularly because of the opposing downregulation of TNR at the lesion site in lower vertebrates that show CNS regeneration (Becker et al., 1999), a role of TNR in inhibition of axon growth and regeneration in mammals has been postulated.

1.4.9. TNR interactions

As for TNC several receptors and interaction partners have been identified for TNR (Table2).

	Interaction partner	**Reference**
Cell surface receptors:	CAMs including neurofascin, contactin / F3, myelin associated glycoprotein (MAG)	Weber et al., 1996; Xiao et al., 1996, 1997; Volkmer et al., 1998; Yang et al., 1999
	Integrins	Jones and Jones, 2000
	CALEB	Schumacher and Stube, 2003
	Phosphacan	Xiao et al., 1996, 1997
	Disialogangliosides	Probstmeier et al., 1999
	Voltage-dependent sodium channels	Srinivasan et al., 1998
ECM interactions:	Aggrecan, versican, brevican, neurocan, fibronectin	Milev et al., 1997; Weber et al., 1996; Volkmer et al., 1998; Pesheva et al., 1994; Probstmeier et al., 1999; Jones and Jones, 2000

Table 2 Summary of TNR interactions

The multidomain and oligomeric structure of the ECM glycoproteins TNR and TNC suggests that they may serve to link cell surface molecules between different cells and to the ECM network.

1.5. Aims of study one

- TNs represent a prominent molecular family of the extracellular matrix with diverse functional implications such as adhesion, migration, differentiation and neurite outgrowth. The major aim of the first study was to investigate whether the TNs TNC and TNR play a role in modulating NSC behaviour *in vitro* and possibly can improve NSC abilities in respect to a therapeutic use.

- NSCs hold a potential for therapeutic use in degenerative diseases of the nervous system. Several studies investigated this potential by injecting cells into different brain regions of rodents. As part of the TN analysis we aimed to establish a co-culture system of hippocampal slices and NSCs to enable a simple *in vitro* analysis of applied NSCs that resembles the *in vivo* conditions after insertion of stem cells into living animals.

- Analysis of TNC deficient mice gave some insight into TNC function in development and plasticity of the nervous system. However, the results described in the literature are not fully conclusive, often contradictory and underlying mechanisms are open. To further elucidate TNC's role in nervous system development a transgenic mouse should be constructed that over-expresses TNC in astrocytes.

2.0 Materials and Methods

2.1. Reagents, disposables, instruments

If not itemized in this paragraph, origin of materials and instruments is referenced in the corresponding sections. All chemicals were obtained from the following companies in *proanalysis* quality: Amersham Pharmacia Biotech (APB, Freiburg, D), Bio-Rad (Munich, D), Invitrogen (Karlsruhe, D), Carl Roth (Karlsruhe, D), Merck (Darmstadt, D), Serva (Heidelberg, D) and Sigma-Aldrich (Deisenhofen, D). Molecular cloning reagents were obtained from Ambion (Cambridge, UK), APB (Freiburg, D), BD Biosciences Clontech (Heidelberg, D), Promega (Mannheim, D), Qiagen (Hilden, D) and Stratagene (Amsterdam, NL). DNA and RNA purification kits were purchased from APB (Freiburg, D) and Qiagen (Hilden, D). Nucleic acid molecular weight markers were purchased from NEB (Frankfurt a. M., D). Oligonucleotides/primers were synthesized by MWG biotech AG (Ebersberg, D) or Metabion (Planegg, Martinsried, D). Restriction endonucleases were obtained from AGS Hybaid (Heidelberg, D), MBI Fermentas (St. Leon-Rot, D) and NEB (Frankfurt a. M., D).
Cell culture products were obtained from Invitrogen (Karlsruhe, D) or Biochrom (Berlin, D).
Centrifugations were performed using RC50*plus* with SLA3000, SLA 1500, SA600 (Sorvall, Kendro, Hanau, D), the microcentrifuge 5415D or bench-top centrifuges 5417R and 5403 (Eppendorf, Hamburg, D).

2.2. Bacterial media

All media were autoclaved prior to use.

Luria Bertani broth (LB per litre H_20)	10 g NaCl 10 g tryptone or peptone 5 g yeast extract In H_20, pH 7.0 with 5 N NaOH (optional)
LB agar (per litre H_20)	10 g NaCl 10 g tryptone or peptone 5 g yeast extract 20 g agar In H_20, pH 7.0 with 5 N NaOH (optional)

2.3. Buffers and stock solutions

Buffers and solutions are listed below. All more method-specific solutions are specified in the accompanying sections.

DEPC-H2O	0.1 % (w/v) diethylpyrocarbonate autoclaved after stirring overnight
Phosphate buffered saline (PBS 10x, Morphology)	1.36 M NaCl 0.1 M Na_2HPO_4 27 mM KCl 18 mM KH_2PO_4 In H_20, pH 7.4
PBST	0.1 % (v/v) Tween 20 in 1x PBS
4 % Paraformaldehyde (PFA)	4 % (w/v) paraformaldehyde dissolved at 60 °C under stirring in 1x PBS

Saline sodium citrate buffer (SSC, 20x)	3 M NaCl 0.3 M *tri*-sodium citrate In H_20, pH 7.4
Sodium Chloride-Sodium Hydrogen Phosphate-EDTA (SSPE, 20x, for 1l H_20)	3 M NaCl 0.2 M Na_2HPO_4 x H_2O 0.02 M EDTA In H_20, pH 7.4 with NaOH

2.4. Molecular biological methods

If not otherwise indicated, standard molecular biological techniques were carried out as described (Sambrook et al., 1989).

2.4.1. Maintenance of bacterial strains

Strains were stored as glycerol stocks (LB-medium, 25 % (v/v) glycerol) at – 80 °C. To regrow particular strains, an aliquot of the stock was streaked on an LB-plate containing the appropriate antibiotics and incubated overnight at 37 °C. Plates were stored up to 6 weeks at 4 °C.

2.4.2. Production of competent bacteria

Bacteria (*E. coli* DH5α, Invitrogen or NR 3704 (dam-) Institute Prof. Schachner) were streaked on LB-agar dishes and grown overnight at 37 °C with constant shaking. Fifty ml of LB broth were inoculated with 5 colonies and grown at 37 °C under constant shaking (> 200 rpm) until the culture had reached an optical density (OD_{600}) of 0.35-0.45. Growth of bacteria was stopped by a 5-min incubation step on ice. Cells were pelleted at 1000 g for 15 min (4 °C) and – after removal of the supernatant – resuspended in 17 ml prechilled (4 °C) RF1 solution (see below). Following 15-min incubation on ice, the centrifugation was repeated. The cell pellet was resuspended in 4 ml prechilled (4 °C) RF2 (see below) and incubated again for 15 min on ice. Bacteria

were frozen as 50 – 100 µl aliquots in liquid nitrogen and stored at – 80 °C. Transformation capacity / efficacy of cells was tested by a transformation with a distinct quantity (pg-ng) of purified supercoiled plasmid DNA.

RF1	**RF2**
100mM RbCl	10 mM MOPS (pH 6.8)
50 mM MnCl2	10 mM RbCl
30 mM KOAc	75 mM CaCl2
10 mM CaCl2	150 g/l glycerol

The pH of the solution was adjusted to 5.8 with 0.2 M acetic acid.

2.4.3. (Re-) Transformation of DNA into bacteria

10 ng of plasmid DNA or 20 µl of a ligation mixture were added to 100 µl of competent DH5α and incubated for 30 min on ice. After a heat shock (2 min, 42 °C) and successive incubation on ice (3 min), 800 µl of LB-medium were added to the bacteria and incubated at 37 °C for 30 min with constant agitation. Cells were collected by centrifugation at 1000 g for 2 min at RT. The transformed cells were plated on LB plates containing the appropriate antibiotics and incubated at 37 °C for 12 - 16 h to allow single colonies to grow.

2.4.4. Purification of nucleic acids

Plasmid DNA purification from bacterial cultures

Mini-scale plasmid isolation

Three ml LB/Amp-Medium (100 µg/ml ampicillin) were inoculated with a single colony and incubated overnight at 37 °C with constant agitation. Cultures were transferred into 2-ml Eppendorf tubes and the cells were pelleted by centrifugation (12 000 rpm, 1 min, RT). Plasmids were isolated from the bacteria using the GFX *micro* plasmid prep system (APB), according to the manufacturer's protocol. The DNA was eluted from the columns by addition of 50 µl Tris-HCl (10 mM, pH 8.0) with subsequent centrifugation (12 000 rpm, 2 min, RT). Plasmid DNA was stored at - 20°C.

Plasmid DNA isolation from 15-ml cultures

To rapidly obtain higher amounts of DNA, the Macherey-Nagel Nucleospin kit was used. Fifteen ml LB/Amp-Medium (100 µg/ml ampicillin) were inoculated with a single colony and incubated overnight at 37 °C with constant agitation. Cultures were transferred into 15-ml Falcon tubes and the cells were pelleted by centrifugation (12 000 rpm, 1 min, RT) in an Eppendorf centrifuge. Plasmids were isolated from the bacteria according to the manufacturer's protocol with the exception that twice the suggested amount of buffer was used. DNA was eluted from the columns by adding 50 µl of prewarmed (70 °C) Tris-HCl (10 mM, pH 8.0) with subsequent centrifugation (12 000 rpm, 2 min, RT) twice. Finally, the DNA concentration was determined as described in 2.6.14.

Plasmid DNA isolation from 500 ml cultures (Maxipreps)

For preparation of large quantities of DNA, the Qiagen Maxiprep kit was utilized. A single colony was inoculated in 2 ml LB/amp (100 µg/ml ampicillin) medium and grown at 37 °C for 8 h with constant agitation. Afterwards, this culture was added to 500 ml LB/amp medium (100 µg/ml ampicillin) and the culture was incubated at 37 °C with constant agitation overnight. Cells were pelleted (6 000 g, 15 min, 4 °C) and DNA was isolated as described in the manufacturer´s protocol. Finally, the DNA pellet was resuspended in 600 µl of prewarmed (70 °C) Tris-HCl (10 mM, pH 8.0) and the DNA concentration was determined (see 2.6.14).

DNA fragment purification

For purification of DNA fragments the silica matrix-based High Pure PCR-Purification kit (Roche, Mannheim, D) was used according to the manufacturer's protocol. The DNA was eluted from the column by addition of 50 µl prewarmed (70 °C) Tris-HCl (10 mM, pH 8.0). The DNA concentration was determined using the undiluted eluate.

DNA fragment extraction from agarose gels

For isolation and purification of DNA fragments from agarose gels, ethidiumbromide-stained gels were illuminated with UV-light and the appropriate DNA band was excised from the gel with a clean scalpel and transferred into an Eppendorf tube. The fragment

was isolated utilizing the silica matrix-based QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol. The fragment was eluted from the column by addition of 50 µl pre-warmed (70 °C) Tris-HCl (10 mM, pH 8.0). The DNA concentration was determined using the undiluted eluate.

2.4.5. DNA agarose gel electrophoresis

To analyze restriction digestions, quality of nucleic acid preparations, etc., horizontal agarose gel electrophoresis was performed. Gels were prepared by heating 0.8 - 2.5 % (w/v) agarose (Invitrogen, electrophoresis grade) in Tris-acetate buffer (TAE, 40 mM Tris-acetate, 2 mM EDTA, pH 8.0), depending on the size of fragments to be separated. DNA samples were adjusted to 1 x DNA sample buffer (50 % glycerol in TAE, 0.025 % Orange G dye, Merck) and were subjected to electrophoresis at 10 V/cm in BIO-Rad gel chambers in 1 x TAE running buffer. Afterwards, gels were stained in 0.5 µg/ml ethidiumbromide in 1 x TAE solution for approximately 20 min at RT. Thermo-photographs of transilluminated gels were taken, or bands were made visible on an UV-screen (λ = 360 nm) (E.A.S.Y. UV-light documentation; Herolab, Wiesloh, D) and desired fragments were cut out with a scalpel. Extraction of DNA fragments from agarose plugs is described above.

2.4.6. Sequencing of DNA

Sequence determination of dsDNA was performed by Dr. W. Kullmann and M. Daeumigen at the sequencing facility of the ZMNH. Fluorescencedye-labelled chain-termination products (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Wellesly, MA, USA) were analyzed with an ABI Prism 377 DNA Sequencer (Perkin Elmer). For preparation, 0.8 - 1 µg of DNA was diluted in 7 µl double-distilled (dd) H_20 and 1 µl of the appropriate sequencing primer (10 pM) was added.

2.4.7. Phenol/chloroform extraction

An equal volume of a neutralized phenol / chloroform solution (1:1) was added to the aqueous DNA-containing sample and vortexed for 1 - 2 min (longer for larger volumes)

to create an emulsion. To avoid shearing, samples containing genomic DNA were only gently mixed. After centrifugation at 16 000 g (RT) for 2 - 5 min, the aqueous top layer was carefully transferred to a new tube, avoiding any flocculent material at the interface. The lower, organic phase was extracted with an equal volume of TE buffer for optimal recovery. In order to remove residual phenol, the combined aqueous phases were extracted with an equal volume of chloroform. After centrifugation at 16 000 g (RT) for 5 min, the aqueous top layer was carefully transferred to a new tube, designated for further DNA purification to precipitation (see 2.6.8).

2.4.8. Precipitation of DNA

The salt concentration of aqueous DNA solutions was adjusted by adding 1/10 volume of sodium acetate, pH 5.2. Cold ethanol (- 20 °C, 2.5 volumes) was then added and the samples were mixed well. Following incubation on ice for 30 min, the samples were centrifuged for 15 min (16 000 g, RT). For optimal purity, the pellet was loosened from the tube during inverting and was broken up in ethanol. After removal of the supernatant, a quick (1 - 2 s) centrifugation step was performed and residual ethanol was aspirated. Supernatants were removed and DNA pellets air dried (approximately 5 min at RT). The DNA was resuspended in an appropriate volume of prewarmed water.

2.4.9. Precipitation of RNA

The salt concentration of aqueous RNA solutions was adjusted by adding 1/2 volume of ammonium acetate, pH 7.5. Then 2.5 volumes of cold ethanol (- 20 °C) were added and the samples were mixed well. Following incubation at – 20 °C for 1 - 12 h, the samples were centrifuged for 30 min (12 000 g, 4 °C). For optimal purity, the pellet was loosened from the tube during inverting and was broken up in – 20 °C cooled 80 % ethanol. After removal of the supernatant, a quick (1 – 2 s) centrifugation step was performed and residual ethanol was aspirated. Supernatants were removed and RNA pellets air dried shortly and resuspended in an appropriate volume of RNAse-free water.

2.4.10. Enzymatic manipulation of DNA

Restriction of DNA

Restriction enzyme digestions were performed by incubating dsDNA molecules with an appropriate amount of restriction enzyme(s), the respective buffer as recommended by the supplier(s), and at the optimal temperature for the specific enzyme(s), usually at 37 °C. In general, 20 µl digests were planned. For preparative restriction digests, the reaction volume was scaled up to 100 µl. Digestions were composed of DNA, 1 x restriction buffer, the appropriate number of units of the respective enzyme(s) (due to glycerol content the volume of the enzyme(s) added should not exceed 1/10 of the digest volume), and the sufficient nuclease-free H_2O to bring the mix to the calculated volume. After incubation at the optimal temperature for a reasonable time period (mostly 2 - 3 h or overnight), digests were stopped by incubation for 20 min at 65 °C. If reaction conditions of enzymes were incompatible to each other, DNA was digested successively with the individual enzymes. Between individual reactions, DNA was purified (see 2.4.4).

Plasmid DNA fragments

For cloning of distinct regions of plasmid DNA, donor molecules were digested with appropriate restriction enzyme(s). Even though direct ligation using DNA from inactivated restriction digest was possible, mostly complete digests were applied to agarose gel electrophoresis. Appropriate bands were cut out and DNA was eluted from agarose plugs, thus avoiding unwanted by-products during subsequent ligation reactions. Non-complementary overhanging ends were converted to blunt ends prior to ligation using the Klenow enzyme.

Enzymatic manipulation of vector DNA prior to ligation

When used as vectors, plasmids were digested at one locus either by a single restriction enzyme or by two at a multi-cloning site to achieve insertion of target DNA in a defined orientation. Digestion reactions were carried out using 5 - 10 µg of plasmid DNA as starting material. When digestions were verified as complete and correct by agarose gel electrophoresis, complete restriction digests were subjected to preparative agarose gel electrophoresis and appropriate bands representing digested vectors were cut out and

vector DNA was extracted from agarose plugs. To prevent self-circularization of blunt end DNA by DNA ligase, SAP buffer (Boehringer Ingelheim) and 1 U SAP (shrimp alkaline phosphatase) per 100 ng plasmid DNA were added to remove 5´-phosphates. The probe was incubated at 37 °C for 2 h and the reaction was terminated by incubation at 70 °C for 10 min. The plasmid DNA was used for ligation without further purification.

Introduction of additional restriction sites

Frequently the vector did not contain suitable restriction enzyme sites for insertion of DNA. In such cases, additional restriction enzyme sites were introduced into the vector by insertion of polylinkers, short base fragments containing sequences for restriction enzymes. Prior to ligation with vector DNA the polylinkers in 5'-3'and reverse 3'-5' direction were hybridized using 100 pM each heated for 30 min at 75 °C and slowly cooled down. Hybridized polylinker was used for ligation at 200 pM.

Ligation of plasmid vector and insert DNA

Ligation of DNA fragments was performed by mixing 50 ng vector DNA with the fivefold molar excess of insert DNA. One µl of T4-Ligase and 2 µl of ligation buffer (Roche) were added and the reaction mix was brought to a final volume of 20 µl. The reaction was incubated either for 2 h at room temperature (sticky ends) or overnight at 16 °C (blunt ends). The reaction mixture was used directly for transformation without any further purification.

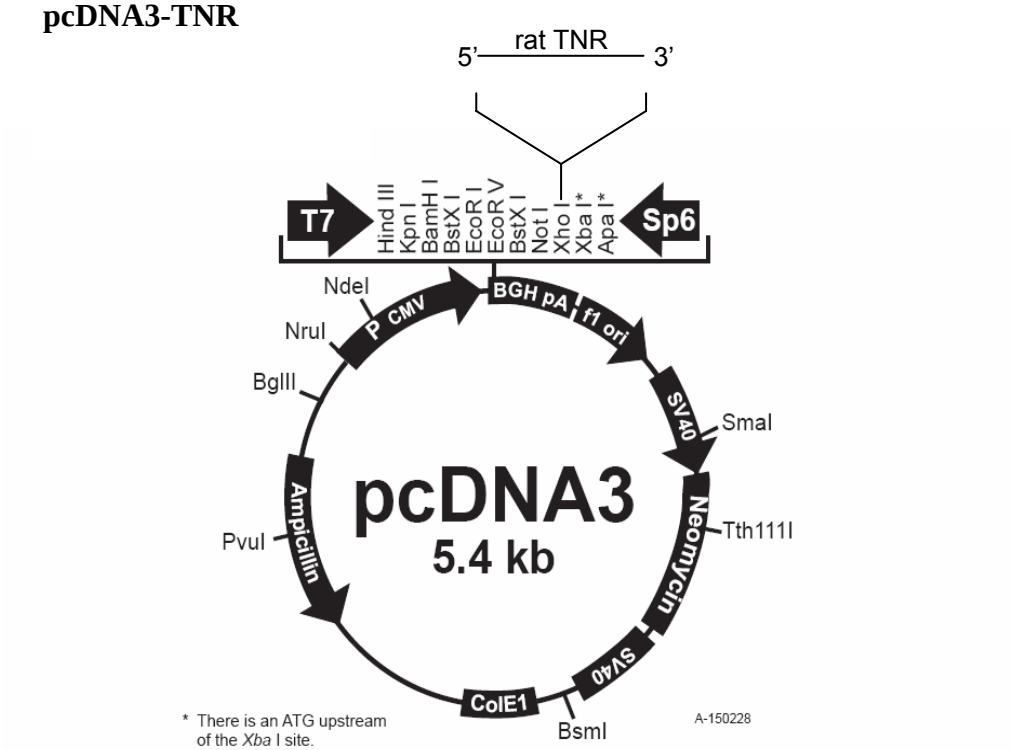
2.4.11. Generation of expression vectors

For the use in neural stem cells two vectors were constructed driving expression of TNR and TNC by the cytomegalovirus (CMV) promoter, designated pcDNA3-TNR and pcDNA3-TNC, respectively (Fig. 4). For the generation of transgenic mice over-expressing TNC in astrocytes, a construct was cloned with the human glial fibrillary acidic protein promoter (hGFAP) driving TNC expression, which was named hGFAP-TNC. The performed cloning steps to derive the separate plasmids are described succeeding.

The **pcDNA3-TNR** expression vector was generated by Dr. Michael Kutsche, by inserting a 6.2 kb SalI rat TNR full-length fragment into the Xho site of the multiple cloning site of the pcDNA3 vector (Invitrogen).
To generate the **pcDNA3-TNC** expression vector, a 6.8 kb EcoRI-SalI cDNA fragment encoding the long mouse TNC splice variant was inserted into the EcoRI-XhoI site of the multiple cloning site of the pcDNA3 vector resulting in a 12.3 kb vector. The extinction of the XhoI site in the vector following ligation was utilized to eliminate residual empty pcDNA3 plasmids by XhoI digestion after ligation. Clones with correctly inserted cDNA were identified by BamHI restriction which results in three fragments of 2.3, 3.1 and 6.8 kb and verified by partial sequence analysis using the following primers:

TNC1: 5'-GAT CAC CTG GTT CAA GCC CT-3'
TNC4: 5'-CCC TCA GCT GGG AGG CTT-3'
TNC9: 5'-GGT TCC ATT GGA AGG GCC AT-3'

pcDNA3-TNR



pcDNA3-TNC

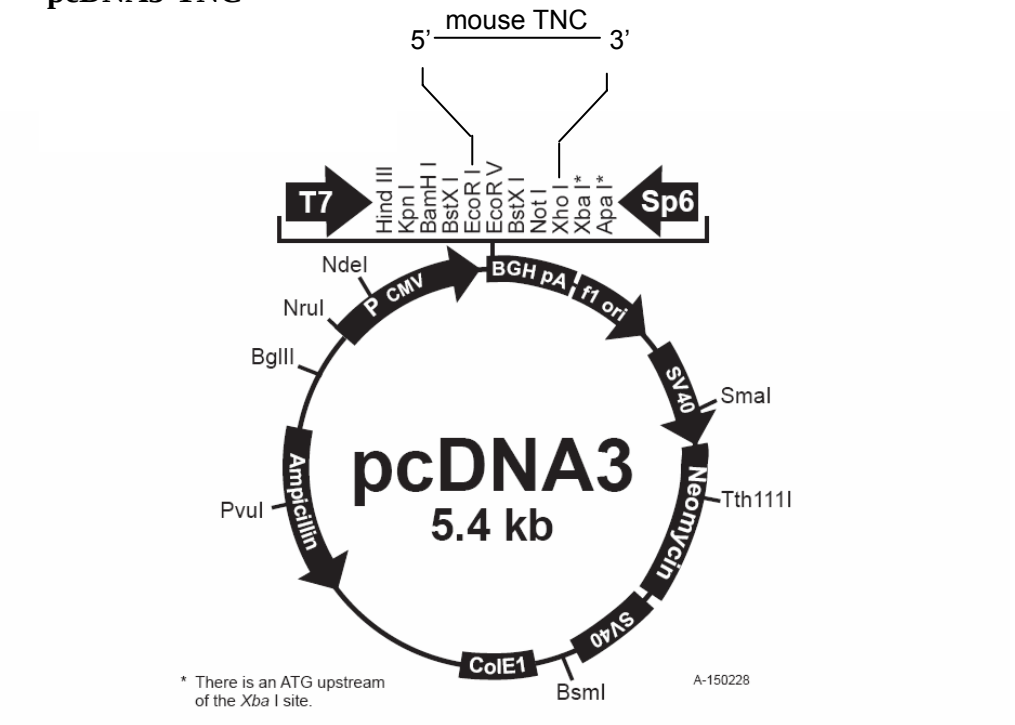


Fig. 4 Vectormaps pcDNA3-TNR and pcDNA3-TNC

Construction of insert cDNA for a transgenic mouse

With the aim to generate a transgenic mouse constitutively expressing the long splice variant of TNC in astrocytes, the full-length long-splice variant of mouse TNC cDNA was cloned behind the hGFAP promoter. The hGFAP promoter has been described to efficiently drive expression of the green fluorescent protein in astrocytes (Zhuo et al., 1997).

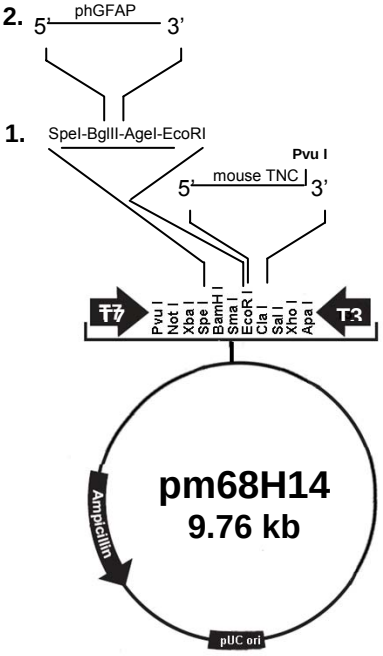


Fig. 5 Vectormap pm48H14-hGFAP-TNC

With the successful strategy the construct (Fig. 5) was derived in two steps.

1. The pm68H14 vector with the desired full-length mouse TNC cDNA (Dr. Michael Kutsche) was modified by insertion of additional restriction sites. A polylinker encoding the restriction sites SpeI-BglII-AgeI-EcoRI was inserted into the SpeI-EcoRI site of the multiple cloning site of pm68H14. Correct insertion was verified by detection of 2 bands at about 3.0 and 6.0 kb after digestion with XhoI and BglII.

2. A BglII-AgeI fragment containing the 2.2 kb hGFAP promoter was inserted into the new AgeI-BglII sites of the modified pm68H14 vector.

Correct ligation was verified by presence of two fragments of about 4.0 and 8.0 kb after digestion with XhoI and partial sequencing using the following primers:

GFAP 4: 5'-GCC CAG TGA ATG ACT CAC CT-3'
GFAP 8: 5'-GGC TGG AGT GGC GCA AAC A-3'

To generate transgenic animals the 9.0 kb hGFAP-TNC fragment was excised from the vector with PvuI and submitted to pronucleus injection; 15 µg DNA were employed for digestion. The fragment was then eluted from a preparative gel using the gel extraction kit from Machery and Nagel and the concentration was adjusted to 100 µg/ml.

2.4.12. Pronucleus injection

The hGFAP-TNC transgene was injected into the pronuclei of 1-cell stage mouse embryos which were subsequent implanted into pseudo-pregnant females.
The injections were performed by Dr. I.Hermans-Borgmayer at the transgene facility of the ZMNH. Mice that showed transgene insertion into their genome were identified by Southern blot analysis and PCR.

2.4.13. Preparation of genomic DNA

To determine the genotype of individual animals, a tail-tip biopsy was incubated in 400 µl Boston buffer (50 mM Tris-HCl, 50 mM KCl, 2.5 mM EDTA, 0.45 % NP40, 0.45 % Tween 20, pH 8, 0.1 mg/ml proteinase K) overnight at 55 °C. The tissue lysate was vortexed to shear the genomic DNA and centrifuged for 2 min at 12 000 g at RT. One µl of the supernatant, containing a crude preparation of genomic DNA, was subjected to a standard polymerase chain reaction (PCR) with appropriate primer combinations under stringent conditions.
A standard PCR was also used to identify transgenic mice, but prior to PCR the genomic DNA of the lysate after digestion with Boston buffer was cleansed either by chloroform–phenol extraction or by usage of the DNA Isolation Reagent for genomic DNA (AppliCem, Darmstadt, D) following the supplier's instructions.

2.4.14. Total RNA isolation from mouse brain tissue

Total RNA from brain tissue was purified using the Trizol (Invitrogen) reagent. The mouse brain was quickly isolated, frozen in liquid nitrogen and ground with a liquid nitrogen cooled mortar. Brains from young mice (up to 5 days old) were processed in one 15 ml falcon tube, brains from older mice (up to 13 weeks of age) - in two and older brains - in three tubes. Per tube 3 ml Trizol were added and the probe was incubated for minimum 5 min at RT until the tissue was completely dissolved. After addition of 300 µl chloroform, vortexing and incubation at RT for 3 min, the contents of one tube was divided into two 2-ml Eppendorf tubes and centrifugated at 10 000 rpm for 15 min (RT). The upper phase was transferred into a new 2-ml tube without disturbing the

interphase. Following addition of 750 µl isopropanol, vortexing and incubation at RT for 10 min, the RNA was pelleted by centrifugation (10 000 rpm, 10 min, RT). The supernatant was removed, the pellet air-dried only shortly and resuspended in RNAse-free water. The amount of water varied with the number of tubes initially employed. For one tube, 500 µl of water were added per pellet, for 2 tubes - 250 µl and for three tubes - 133 µl. Subsequently, all samples of one original probe were combined and purified using the silica-gel-membrane technology as adopted in Qiagen´s RNeasy system following the manufacturer's instructions for a RNA clean-up. All buffers used were provided by the manufacturer. Finally, total RNA was eluted in 250 µl RNAse-free water. Integrity of the purified total RNA was assessed by spectrophotometry (scan from $\lambda = 200 – 350$ nm). Total RNA samples were stored at – 80 °C.

2.4.15. Photometric quantification of nucleic acids

DNA, RNA and oligonucleotide concentrations were estimated in aqueous solutions by measuring adsorptions at $\lambda = 260$ nm against blank probes (Spectrophotometer Ultrospec 3000 / DPV-411 printer; APB, Freiburg, D).
The determined optical densities (OD) values were multiplied by 50, 40 and 30 for double-stranded (dsDNA, RNA and oligonucleotides, respectively, to calculate concentrations in g/ml.
Interference by contaminants was recognized by calculation of ratios. The ratio A260 / 280 was used to estimate the purity of nucleic acid, since proteins absorb at 280 nm. Pure DNA should have an A250 / 280 ratio of 1.8, whereas pure RNA should give a value of approximately 2.0. Absorption at $\lambda = 230$ nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. In the case of pure samples, the ratio A260 / 230 should be approximately 2.2.

2.4.16. Nucleic acid amplification

The *in vitro* amplification of DNA fragments using PCR was usually performed in a MJ PTC-200 DNA ENGINE_ classic thermal cycler. Routinely, PCR reactions were set up by adding the following ingredients into a 0.2-ml thin-walled tube (Biozym Diagnostik GmbH, Hessisch-Oldendorf, D): the template DNA (typically plasmid or first strand

cDNA), the primers flanking the region(s) to be amplified, dNTPs, buffer and DNA polymerase. Primer sequences were selected manually or electronically determined with the PrimerSelect software from the Lasergene software suite (DNASTAR, WI, USA). The volume of the mixture per single PCR was typically 20 - 50 µl. For general PCR reactions Taq DNA polymerase (Invitrogen Karlsruhe, D) and for RT-PCR HotStarTaq DNA polymerase (Qiagen, Hilden, D) was used. Cycling parameters are mentioned in the context of enzyme use. Number of cycles required for optimum amplification varied between 25 and 40 depending on the amount of starting material and the efficiency of each amplification step. A final incubation step at the extension temperature ensured fully double stranded molecules from all nascent products. Following cycling, aliquots (typically 5 - 10 µl) were analyzed by agarose gel electrophoresis to detect the amplified products.

Single colony PCR

The ligation of DNA fragments at low concentrations is a rare event and identification of desired clones can be time consuming. To screen large amounts of bacterial colonies for the desired insert, single colonies were picked from a transformation plate with a sterile tooth pick and dotted onto a new LB plate. The rest of the colony on the tooth pick was lyzed in 30 µl Boston buffer lacking proteinase K (50 mM Tris-HCl, 50 mM KCl, 2.5 mM EDTA, 0.45 % NP40, 0.45 % Tween 20, pH 8) at 55 °C for 1 h. To test for the presence of the insert DNA, 2 µl of this lysate were subjected to a standard PCR with appropriate primers. For plasmid preparation, the colony that contained the desired vector was inoculated into LB medium with the colony dotted on the LB plate.

Genotyping by PCR

For genotyping of mice, 1 µl of genomic DNA was used for a standard PCR using the appropriate primer combinations under stringent conditions. To distinguish between different possible genotypes (homozygous, heterozygous and wild-type or transgenic), two primers were constructed which either flanked the mutated region or, in case of a transgene, lay within the inserted DNA fragment. Thus, the PCR gave rise to three different band patterns, as visualized by agarose gel electrophoresis, and each pattern represented one genotype.

For identifying transgenic hGFAP-TNC animals the following primers were used:

Primer 1, hGFAP, lies within the promoter region:
5'-GTA CCA CCT GCC TCA TGC AG-3'
Primer 2, TNCdn224, lies within the TNC coding region of the transgene:
5'-CCT GAT TTT CTT GAG GAC CC–3'
Amplicon: 605bp. As a control for DNA content the wild-type CD24 PCR was routinely performed in parallel (see materials and methods part2).

Reaction mix:

1 µl genomic DNA
1 µl 10 pmol primer 1
1 µl 10 pmol primer 2
1 µl 10mM dNTPs
2 µl 10x-PCR-Puffer
2 µl $MgCl_2$ (1mM)
0.5 µl Taq
ad 20µl

PCR protocol:

Cycle 1	94 °	5 min
2	94 °	1 min
3	62 °	30s
4	72°	2 min
2-4		36 repeats
5	72°	10 min

RT-PCR

For reverse transcription *in vitro*, the RNA-dependent DNA polymerase activity and a hybriddependent exoribonuclease (RNaseH) activity of the reverse transcriptase enzyme (RT) were utilized to produce single-stranded cDNA from RNA. From 5 µg starting material, first strand cDNA was produced using SuperScriptII (Invitrogen) and hexamer primers following the manufacturer's instructions. The first strand cDNA was used for real-time PCR only if its concentration was ≥ 1µg/µl.
First strand cDNA was stored at – 20 °C or directly used for real-time PCR reactions as described below.

Real-time PCR

To determine the relative amount of TNC mRNA, real-time PCR was performed using the SYBR Green I method (Stratagene) following the manufacturer's instructions. This method allows the quantification of a template during the exponential phase of amplification based on fluorescence measurements (7900 Light Cycler; Applied Biosystems, Foster City, CA). SYBR GreenI is a non-specific DNA-binding dye that emits fluorescence light when bound to dsDNA. The fluorescence intensity increases with each amplification cycle proportionally to the increased amplicon concentration. Hereby the threshold cycle (Ct) value is essential for determination of the first cycle at which the instrument can detect the amplification-generated fluorescence above background level. The Ct value is inversely proportional to the initial copy number. The greater the amount of initial DNA template in the sample is, the smaller the Ct value for that sample will be. The Ct values were used to generate relative comparisons of the change in template concentration among the samples from wild-type animals and transgenic animals.

The cDNA template derived from RT PCR with concentration ≥ 5 µg/ml was diluted 1:10 and subsequently 0.5 µl were used per 20 µl reaction mix. All reactions were performed in triplicates.

The following primers were used:

The amount of initial RNA was normalized with the house-keeping gene hypoxanthine-phosphoribosyl-transferase (HPRT) using the primers:

306f: 5'-GTT CTT TGC TGA CCT GCT GGA-3'
425r: 5'-TCC CCC GTTT GAC TGA TCA TT-3'

The amount of TNC mRNA was determined with the primers:

TNC-6107F: 5'- GCGTTAACTGGTTCCATTGGA-3'
TNC-6217R: 5'-TTTATGCCCGCTTACGCCT -3'

Primer dimerization was controlled by performing melting curve analysis on the PCR products from every run. The Ct values of the target gene TNC and the reference gene

HPRT were between 23 and 25 and 20 - 21, respectively, i.e. well within a recommended range of 10 cycles. Only sample replicates with Ct values differing by less than 0.5 were used for analysis.

2.4.17. Southern blot analysis

To identify mice that had an hGFAP-TNC transgene insertion into their genome, Southern blot analysis was performed.

Genomic DNA from tail biopsies were digested with 50 U SacI overnight, cleansed by chloroform-phenol extraction and subsequent ethanol washes, and separated by electrophoresis in a 0.8 % agarose gel. The DNA was depurinated by incubation of the gel in 0.25 M HCl (15 min, RT) and equilibrated for 15 - 30 min in a solution containing 0.4 M NaOH. The capillary blot procedure was used to transfer the DNA onto Hybond-N(+) membranes (Amersham Pharmacia) by 0.4 M NaOH solution. After transfer for 6 - 8 h the membrane was washed for 10 min in 2x SSC and air-dried. Prehybridization with buffer containing sperm DNA was performed at 65 °C in roller bottles rotated in a hybridization oven for 2 - 4 h.

The probe was derived by SacI cleavage of the hGFAP-TNC vector resulting in a 744 bp DNA fragment that was gel-eluated and its concentration adjusted to 5 ng/µl. Prior to hybridization the probe was radioactively labelled with P^{32} by random priming (see below) and denatured at 95 °C for 5 min before adding to the preheated (65 °C) hybridization solution.

The genomic DNA was hybridized with the radioactively labelled probe in hybridization solution (0.5 ng labelled probe, 10 % PEG 20000, 5x SSPE, 7 % SDS, 1.5x SSPE, 100 µg/ml denaturated salmon sperm DNA) overnight at 65 °C. Unspecifically bound probe was washed from the membrane by washing steps with 2x SSC/0.1 % SDS for 15 min followed by 1x SSC/0.1 % SDS for 30 min and finally 2x 30 min 0.5x SSC/0.1 % SDS at 65 °C. The last washing steps were prolonged until < 30 radioactive decays per s were detected on the membrane. The blot was wrapped in plastic foil and signals were detected by exposure of the membrane for 30 min to Kodak-Xomat-AR film in a cassette equipped with amplifier screens.

2.4.18. Random prime DNA labelling

The cDNA probes used for Southern blot analysis were [^{32}P]-dCTP-labelled with the Ready-to-go DNA labelling beads (Amersham Bioscience). The DNA was first denatured by heating for 3 min at 95 °C and placed immediately on ice for 2 min. Twenty-five ng of the designated cDNA/DNA probe were filled up with DEPC-H_2O to 45 µl and together with 5 µl [^{32}P]-dCTP added to the reaction mix bead. For labelling, mixtures were incubated at 37 °C for 15 min. Directly prior to application of the probes to the prehybridized blots, the molecules were denatured by boiling for 5 min.

2.5. Protein analysis

2.5.1. Protein extraction

Protein extracts were prepared from snap-frozen tissues. High protein concentrations were obtained by homogenization of 30 % (w/v) tissue in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 7.5, 0.2 % NP-40, 1x Complete Protease Inhibitors, Roche Diagnostics) at 4 °C. The homogenate was centrifuged at 20 000 g and 4 °C for 30 min to remove insoluble components. The protein content of the supernatant was determined and appropriate amounts were used for Western blot analysis.

2.5.2. Determination of protein concentration

To ensure comparable protein concentrations in Western blot analysis, the protein concentrations of the extracts were determined in 96 well plates using the Micro BCA Reagent (Pierce) according to the manufacturer's instructions.

2.5.3. Protein analysis by Western blot

Separation of proteins was performed by discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using the Mini-Protean III system (Biorad).

The different proteins were focused on top of the running gel by a 0.8-cm stacking gel containing 5 % (v/v) acrylamide (3.77 ml deionized water, 0.32ml 1 M Tris (pH 6.8),

0.05 ml 10 % SDS, 0.83 ml 30 % acrylamide:bis (29:1), 0.025ml 10 % APS, 0.007 ml TEMED), followed by separation of the proteins along a 4.5-cm running gel containing 10 % acrylamide (3.92 ml deionized water, 5.26 ml 1 M Tris, pH 8.8, 0.14ml 10 % SDS, 4.7 ml 30 % acrylamide:bis (29:1), 0.07 ml 10 % APS, 0.007 ml TEMED). The protein samples were boiled for 10 min in sample buffer (0.312 M Tris-HCl, pH 6.8, 10 % SDS, 5 % β-mercaptoethanol, 50 % glycerol, 0.15 % bromphenol blue) prior to analysis. The samples were then loaded in amounts of 1 ng to 100 µg per lane and the gel was run at constant voltage (80 V) for 10 min followed by 140 V for approximately 1 h until the bromphenol blue reached the end of the gel. The separated proteins were transferred onto a nitrocellulose membrane (Protean nitrocellulose BA 85, Schleicher&Schüll) using the Mini Transblot apparatus (Biorad) according to the manufacturer's instructions. Proteins were blotted electrophoretically at 4 °C in blot buffer (25 mM Tris-HCl, 192 mM Glycin) at constant voltage (80 V for 2 h or 30 V overnight). A pre-stained marker (BenchMark, Invitrogen) was used to determine the molecular weight of the separated proteins and to monitor the electrophoretic transfer. For immunological detection of proteins, nitrocellulose membranes were washed once in TBS (10 mM Tris-HCl, pH 8, 150 mM NaCl) and incubated in 8 ml milk powder solution (2 % in TBS) for 1 h at RT. The antibody was diluted in milk powder solution and incubated for 2 h at RT with constant agitation. Unspecifically bound primary antibody was removed by washing the membrane 5 times in TBS for 5 min each. Secondary antibody was applied in milk powder solution for 1 h and the membrane was washed again 5 times with TBS. Signals were detected using horseradish peroxidase-coupled species-specific secondary antibodies against immunglobulins (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia) on Biomax ML film (Kodak).

2.5.4. Densitometric evaluation of band intensities

The TINA program (Version 2.09, © 1993 Isotoplab University Bochum) was used to analyze relative band densities using digitized scans of developed films from blots stained by indirect immunohistochemistry according to the manufacturer's instructions.

2.6. Cell and tissue preparation and culturing

2.6.1. Animals

Neural stem cell and/or organotypic cultures were preparated from wild-type C57BL/6J mice, C57BL/6J mice ubiquitously expressing the enhanced green fluorescent protein (GFP) under the control of the chicken actin promotor (Okabe et al., 1997) or TNR (Weber e al., 1999) and TNC deficient mice (Evers et al., 2002) and their wild-type littermates.

2.6.2. Neural stem cell culture

For NSC preparation lateral and medial ganglionic eminences were removed from fourteen-days-old embryos and dissociated mechanically with a fire-polished Pasteur pipette in DMEM/F-12 (1:1) (Biochrom) containing glucose (0.6 %), glutamine (2 mM) (Invitrogen), sodium bicarbonate (3 mM) (Invitrogen), HEPES buffer (5 mM), and 20 µl/ml B27 (Invitrogen) and were grown in suspension under which conditions single cells form spherical clones termed neurospheres (Fig. 6). For generation and expansion of neurospheres, EGF (PreproTech, Rocky Hill, NY) and bFGF (or FGF-2) (PreproTech) were added to a final concentration of 10 ng/ml each. The initial seeding density was 200 000 cells/ml. After 6 days *in vitro*, cells were passaged for the first time with a seeding density of 50 000 cell/ml. Cells were separated by incubation with Accutase (Biochrom) for 5 min at 37 °C and subsequent only gentle mechanical dissociation. From the first passage onward, neurospheres were passaged every fifth day. Vital cells were determined by 0.5 % Trypan blue dye (Invitrogen) exclusion. Proliferation and differentiation experiments were performed with EGF-bFGF-generated neurospheres between passages three and six.

2.6.3. Proliferation and differentiation of neural precursor cells

For assessment of overall proliferation of neural precursor cells, bromodeoxyuridine (BrdU) (10 µmol, Sigma-Aldrich) was administered in EGF-bFGF-containing culture medium 2 h before cells were fixed with 4 % PFA.

For assessment of differentiation, neurospheres were mechanically dissociated and plated at a density of 50 000 cells/ml onto 15 mm glass coverslips coated with Poly-l-lysine (PLL, Sigma-Aldrich). This procedure led to equally distributed and well separated single cells that proliferated under the influence of EGF-bFGF until reaching confluency. For differentiation experiments, precursor cells were first maintained in an undifferentiated state for 3 days after plating in EGF-bFGF-containing serum-free culture medium. Growth factors were then removed (omitting any other additives to the medium), and precursor cells were then allowed to differentiate for additional 8 days. Eleven days after plating, coverslips were washed in 1x PBS, and cells were fixed for 30 min in 4 % PFA in 1x PBS.

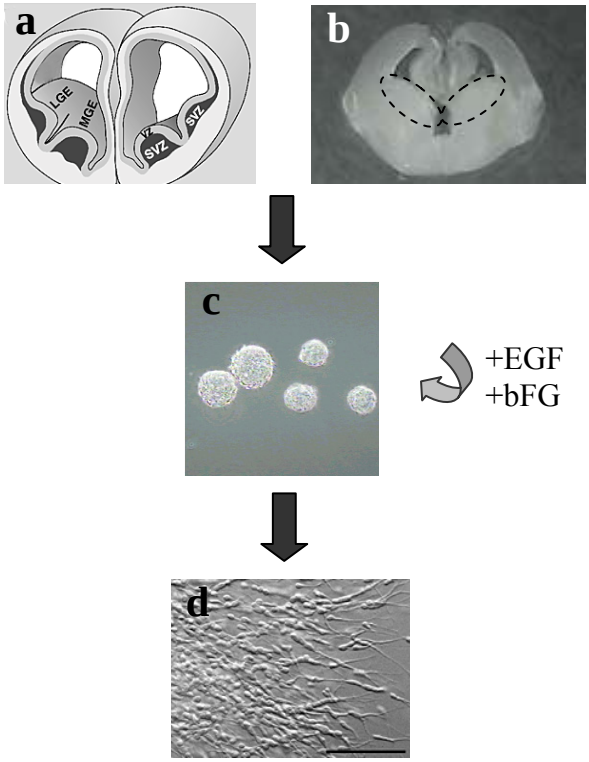
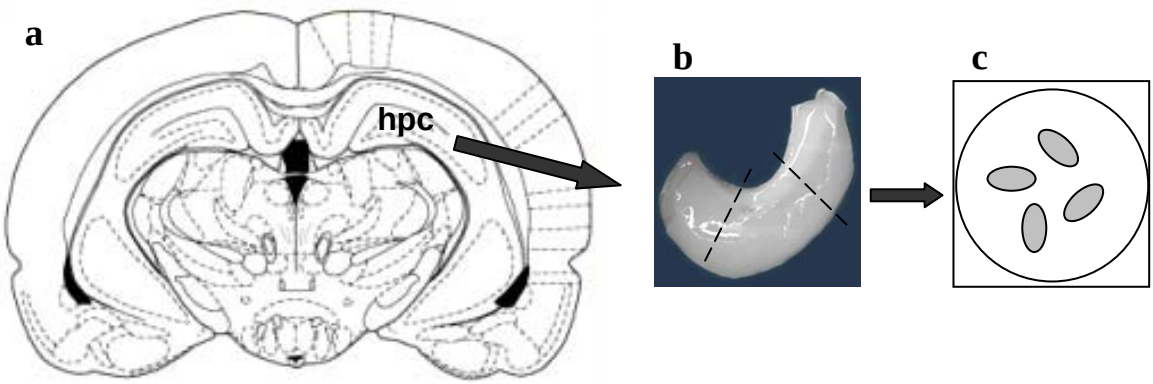


Fig. 6 Preparation and maintenance of neural stem cells
Subventricular zone (SVZ) and ventricular zone (VZ) were dissected from medial and lateral ganglionic eminence (MGE and LGE). **a)** Schematic coronal section through E14 embryonic forebrain. Anterior faces front; dorsal is to the top. **b–d)** Differential interference contrast micrographs. **b)** Isolated ganglionic eminences with adjacent neocortex. Lateral and medial ganglionic eminences that were dissected for neural stem cell cultures are encircled. Under the influence of growth factors EGF and bFGF single cells proliferate forming neurospheres **(c)**. To analyze proliferation and differentiation cells were dissociated and plated onto PLL-coated coverslips or petri dishes **(d).** Scale bar 100 mm.

2.6.4. Preparation of organotypic cultures of hippocampus from rat and mouse

Rat and mouse organotypic cultures were prepared and cultured according to a method developed by Stoppini et al. (1991) (Fig. 7). Tissue slices are cultured on a membrane at the interface between medium and air such that the cultures do not flatten to a monolayer. Rat organotypic cultures were prepared from hippocampi of 8 day old Wilstar rats, mouse cultures from P5 old mice. Hippocampi were dissected on ice and cut vertically in 400 µm slices using a McIllwan tissue chopper. Slices were separated in ice-cold HBSS (Biochrom) and subsequently 4 slices were applied to one Transwell® insert (Corning, Wiesbaden, D) with surplus liquid being sucked away. Inserts were placed into 6-well chambers (Corning) pre-loaded with 1 ml medium and kept at 37 °C and 5 % CO_2. The medium was completely exchanged the next day. Subsequently every other day approximately 800 µl of the medium were exchanged. Beginning on day three of cultivation the preparations were gently washed during media exchange by addition and removal of 400 µl of medium twice.

Cultivation of both rat and mouse slices was initiated with serum-containing media which were gradually replaced by serum-free media during the first week *in vitro*. The rat medium was purchased (CytoGen, Sinn, D). The medium for culturing mouse slices was adopted from Raineteau et al. (2004). The starting medium was composed of 25 % horse serum (Invitrogen), 49 % BME (Eagle) without glutamine (Biochrom), 25 % HBSS, 1 % 200 mM glutamine, 0.6 % glucose, pH 7.2. The serum-free mouse medium consisted of 96.8 % Neurobasal-A (Invitrogen), 2.2 % B27, 0.6% glucose, 1% 200mM glutamine, pH 7.2. By mixing serum-supplemented and serum- free medium in ratios 1:2 and 2:1 media mixes used at day one and day three of the culture period were obtained.



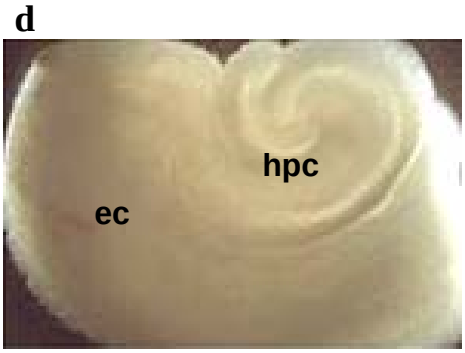


Fig. 7 Preparation of hippocampal organotypic slice cultures The hippocampi of mouse or rat were dissected **(b)** and perpendicularly cut into 400 µm slices. **a)** Schematic drawing of the mouse brain with the hippocampus (hpc). Dorsal is to the top. **b)** Stereoscopic picture of the isolated hippocampus. Only slices from the mid third of one hippocampus were used for culturing, indicated in **b)** as dashed lines. Four slices were applied to one membrane insert **(c)**. **d)** Phase microscopic picture of a rat hippocampal slice cultured for one day. The slice encompasses the hippocampal formation (hpc) and enthorinal cortex (ec).

2.6.5. Transfection of neural stem cells

For transfection of neural stem cells, neurospheres were separated as described for passaging. Cells ($5 – 10x\ 10^6$) were transfected with 10 µg plasmid DNA using the AMAXA Nucleofector kit for mouse neural stem cells (AMAXA, Köln, D) according to the manufacturer's instructions. This type of electroporation yields more than 70 % transfection efficiency for neural stem cells (Richard et al., 2005). Following transfection, the cells were resuspended in 500 µl medium.

2.6.6. Co-culture of hippocampal organotypic slices and neural stem cells

Following the transfection of neural stem cells, cells were counted, centrifugated (1 000 rpm, 5 min) and taken up in 1x PBS to a concentration of 5x 10^5/µl. A volume of 0.1 µl of cell suspension was applied to each organotypic slice using a 1 µl Hamilton syringe (Hamilton, Martinsried, D) that was clamped to a mechanical micromanipulator (Brinkmann Instrumentenbau, Mannheim, D). Twenty-four h after deposition, recipient slices were washed twice to remove unadherent donor cells.
Cells were applied to mouse and rat slices that had been cultured for 8 and 10 days respectively. After additional 6 days mouse slices were washed and fixed in 4 % PFA whereas cells on rat slices were given 10 days to invade or differentiate before fixation.

2.6.7. BHK cell culture and transfection

Baby hamster kidney cells (BHK-21) were cultured in DMEM supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5 % CO2. They were transfected using Metafectene (Biontex, Munich, D) according to manufacturer's instructions.

2.7. Immunohistochemistry

2.7.1. Indirect immunfluorescence on sections

For indirect immunoflouresence, fresh brain tissue was frozen in liquid nitrogen cooled 2-methyl-butan, cut on a cryostat (Cryostat CM3050, Leica Microsystems, Bensheim, D) into 10 – 30 µm - thick sections and mounted on glass slides (Superfrost, Menzel, D). For TNC stainings, cryostat sections were air-dried and surrounded with a Pap-Pen (Kisker, Steinfurt, D) to minimize the amounts of antibodies required for incubation. Other antibody stainings were performed following a protocol described by Irintchev et al. (2005), that is based on a method described by Sofroniew and Schrell (1982) allowing repeated use of antibody solutions (stabilized by the non-gelling vegetable gelatin lambda-carrageenan) in jars and high reproducibility.

Sections were rehydrated in 1x PBS for 10 min and submitted to the general immunohistochemical procedure.

Sections of organotypic cultures were prepared with some adaptations of the general procedure:
Cultured slices were fixed in 4 % PFA for 2 h at 4 °C. Subsequently, the slices were separated from the underlying membrane with the help of a scalpel and cryoprotected in 1x PBS containing 20 % sucrose overnight at 4 °C. Afterwards the slices were mounted flat on coverslips, embedded in TissueTek (Reichert-Jung, Nußloch, D), frozen at – 25 °C and cut on a cryostat (10 µm - or 20 µm-thick sections parallel or vertical to the slice surface, respectively).

2.7.2. Immunohistochemical staining of cultured cells

For indirect immunoflouresence, cultured cells grown on PLL coated coverslips were washed with 1x PBS, fixed in 4 % PFA and washed 3x 15 min in 1x PBS before submitted to immunohistochemical procedures. For incubation with antibodies, 80 µl of solutions were applied to each coverslip.

2.7.3. Indirect immunohistochemistry

Blocking

To prevent unspecific binding of the primary antibodies, tissue sections and cultured cells were incubated in 1x PBS containing 0.2 % Triton X-100, 5 % normal serum (from the species in which the secondary antibody had been raised) and 0.02 % sodium azide for 1 h at RT.

Incubation with primary antibodies

Preparations were incubated with primary antibodies in a humid chamber at 4 °C 1-3 days. Primary antibodies were diluted in 1x PBS containing 0.5 % lamda-carageenan and 0.02 % sodium azide. Sections or coverslips were washed four times (10 min each) with 1x PBS to remove unbound antibodies.

Secondary antibody

To visualize the primary antibodies, fluorescence dye-conjugated secondary antibodies (Dianova, Hamburg, D) were diluted 1:200 in 1x PBS containing 0.5 % lamda-carageenan and applied for 2 h at RT in the dark, washed four times (10 min each) with 1x PBS to remove unbound secondary antibodies. Samples were mounted in anti-fading medium (Fluoromount G, Southern Biotechnology Associates, Biozol, Eching, D) and stored in the dark at 4 °C.

Nuclear staining

In most cases a nuclear counterstain was performed as the last step. Following the third wash sections were incubated 10 min at RT with bis-benzimide solution (Hoechst 33258 dye, 5 µg ml^{-1} in PBS, Sigma-Aldrich, Munich, D).

Antigene retrieval

All antibody stainings except anti-TNC and anti-TNR showed better results with anitigene retrieval prior to blocking.

For antigene retrieval sections were incubated in a pre-heated 10 mM Na citrate solution, pH 9.0 at 80 °C for 30 min.

2.7.4. Analysis

Cell counting

To estimate the total populations of precursor cells, GFP+ cells or bis-benzimide+ nuclei were counted. On every coverslip at least 150 cells were counted in 5 randomly chosen visual fields on a fluorescent microscope (Axiophot 2, Zeiss, Jena, D). Experiments were repeated using at least three independent cultures in duplicates.

The total number of applied neural stem cells on organotypic cultures was obtained by counting GFP+ cells on vertical slices of hippocampal cultures. In case of rat slices, every third 20 µm - thick section from one cultured slice was evaluated for each cell type-specific marker. Since the size of mouse tissue slices and thus number of adherent cells, were smaller than for the rat, all sections from each mouse culture were evaluated. Counting was performed on a laserscanning microscope (Leica). Experiments were repeated with at least three independent cultures.

Double-labelled cells (cell type specific marker / GFP+ or / bis-benzimide+ cells) were counted and the percentages averaged.
A parametric test (Student's t-test for independent samples, two-sided) was used for statistical evaluation. The values are presented as mean values and standard error of the mean (SEM). The accepted level of significance was 5 %.

Documentation
Photographic documentation was made on an a laser scanning microscope (Leica, Wetzlar, D) or on an Axiophot 2 microscope (Zeiss, Jena, D) equipped with a digital camera AxioCam HRC and AxioVision software (Zeiss, image size 1300 x 1030 pixel, RGB mode). The images were additionally processed using Adobe® Photoshop® 6.0 software (Adobe Systems Inc., San Jose, California).

2.8. Antibodies

For **immunocytochemistry** with cell type-specific markers the following **primary antibodies** were used: anti-parvalbumin (PV, mouse monoclonal, clone PARV-19, Sigma-Aldrich, Munich, D, dilution 1:1000), anti-NeuN (mouse monoclonal, clone A60, Chemicon, Hofheim, D, 1:1000), anti-cyclic nucleotide phosphodiesterase (CNPase, mouse monoclonal, clone 11-5B, Sigma-Aldrich 1:1000) anti-classic β-tubulin (mouse monoclonal, Sigma-Aldrich 1:400) and (rabbit polyclonal, Convance, Berkeley, CA, 1:2000,), anti-glial fibrillary acidic protein (GFAP, mouse monoclonal, Sigma-Aldrich, 1:1000), anti-S-100 (rabbit polyclonal, purified IgG fraction, DakoCytomation, Hamburg, D, 1:500), anti-nestin (Developmental Studies Hybridoma Bank, Iowa City, IA, 1:50), anti-calbindin (CB, rabbit polyclonal, affinity purified, Sigma-Aldrich, 1:1000), anti-myelin-basic protein (MBP, rat monoclonal, Chemicon, 1:400), anti-neuronal protein gene product 9.5 (pGp 9.5, Biotrend, Cologne, D, 1:500), The mouse monoclonal antibody 619 raised against TNR has been previously described (Pesheva et al., 1989, used as supernatant diluted 1:10). The rabbit polyclonal antibodies KAF 9-2, KAF14 and pk7 have been previously described (Bartsch et al., 1994) and used at dilutions of 1:100, 1:500 and 1:200, respectively.

For detection of the primary antibodies, appropriate cyanine- (Cy2), indocarbocyanine- (Cy3) and indodicarbocyanine- (Cy5) conjugated **secondary antibodies** (Dianova, Hamburg, D) were used.

For **BrdU** staining, DNA was denatured with 70 % ethanol for 5 min at RT and with 2.4 M HCl for 10 min at 37 °C. Monoclonal mouse antibody to BrdU (Sigma-Aldrich, 1:200) was administered overnight at 4°C.

Reliability of all primary antibodies was monitored by including appropriate positive controls in each experiment. For negative controls, primary antibody was omitted.

For immunochemical detection in **Western blots** the following **primary antibodies** were used: polyclonal rabbit antibody to epidermal growth factor receptor (EGFR, Santa Cruz Biotechnology, Santa Cruz, CA, 1:400), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, mouse monoclonal, Chemicon, 1:4000), anti-phosphorylated Erk (P-Erk, mouse monoclonal, Santa Cruz Biotechnology, 1:2000), anti-Erk (rabbit polyclonal, Santa Cruz Biotechnology, 1:4000), anti-amyloid precursor protein (APP, mouse monoclonal 22C11, Chemicon,1:10000), anti-TNC (rabbit polyclonal, KAF9-2, described in Bartsch et. al 1994, 1:10000), anti-TNR (mouse monoclonal, clone 596, described in Pesheva et al. 1989, 1:10) As **secondary antibodies** horseradish peroxidase-conjugated antibodies (HRP, diluted 1:200) to rabbit, rat or mouse were used (Dianova).

3.0. Results

3.1. TN functions in neural stem behaviour

3.1.1 Immunohistochemical detection of TNR and TNC in neural stem cell cultures

With the aim to study the effects of TNR and TNC on neural stem cell development we first investigated their expression by cultured neural stem cells. Neural stem cells from the embryonic mouse striatum, the ganglionic eminences, were cultured in suspension admitting neurosphere formation. After three to five passages neurospheres were dissociated and plated on PLL-coated coverslips. Addition of the growth factors EGF and bFGF to the culture medium induces proliferation and the cells maintain an undifferentiated state as can be monitored by nestin expression. Under such conditions TNC but not TNR protein expression could be detected by antibody staining of the cells adherent to coverslips. As expected for a secreted molecule TNC expression was not visible in the somata but appeared to accumulate around cell bodies resembling net like structures (Fig. 17d). Expression of TNR *in vivo* starts later than of TNC, in mice

around birth and is associated with differentiating cells such as oligodendrocytes (Bartsch, 1996). We tested whether TNR will be detectable in our culture after induction of differentiation by withdrawal of growth factors. Two days after growth factor withdrawal TNR positive patches became visible. As observed for TNC the expression appeared interstitially surrounding clusters of cells (Fig. 8).

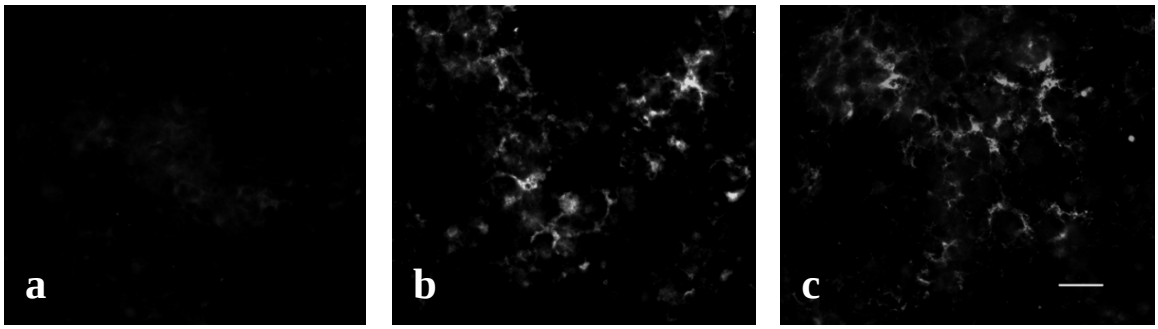


Fig. 8 TNR expression of neural stem cells
Dissociated neural stem cells adherent to PLL-coated coverslips were induced to differentiate by growth factor withdrawal and analyzed for TNR expression by immunohistochemical detection at the day of plating **(a),** 2 days **(b)** - and 4 days **(c)** after induction of differentiation. After 2 days TNR positivity becomes visible and the protein appears to accumulate between cell clusters. Scale bar: 40 µm.

TNR and TNC are both secreted molecules thus the entire cell population of the culture may be influenced by their expression. We may learn about the role of TNs during early developmental events such as differentiation and proliferation of neural stem cells by looking at the effects their deficiency exhibits on neural stem cells in this culture system.

3.1.2. Impact of TN deficiency on neural stem cell differentiation *in vitro*

Striatal neural stem cells were prepared from TNR and TNC deficient and corresponding wild-type mice embryos. Eight days after differentiation of the cultured stem cells was induced by withdrawal of the growth factors EGF and bFGF the

differentiation into different cell lineages was analyzed by immunostaining with neural cell type specific antibodies (Fig. 9+10). The total number of cells was obtained by nuclear bis-benzimide staining. At the end of the differentiation period the majority of neural stem cells was found to have differentiated into astrocytes with a typical flat and broaden morphology, a large nucleus and GFAP positive intracellular fibers (mean values range from 34.7 % to 53.2 %). In TNR+/+ and TNR-/- cultures comparable percentages of cells exhibited GFAP positivity (TNR+/+: 53.15 % ± 1.6; TNR-/-: 49.77 % ± 7.6; ttest: $p > 0.05$; $N = 4$). With 45.57 % (± 2.7) the TNC deficient cells showed an increased probability to differentiate into astrocytes compared to their TNC+/+ littermate cells (34.74 % ± 3.3; ttest: $p = 0.04$; $N = 4$). A smaller percentage of cells, below 13 %, acquired a neuronal fate as revealed by staining with an antibody for the neuron specific β-tubulin. TNR deficiency did not lead to differences in the percentage of neurons in comparison to cultures of wild-type animals. Although the mean value of neurons in the TNR-/- culture was only approximately half compared to the TNR+/+ culture, the difference did not reach significant levels (TNR+/+: 4.51 % ± 1.2; TNR-/-: 2.2 % ± 0.5; ttest: $p > 0.05$; $N = 4$). An additional staining with the pan-neuronal marker pGp9.5, confirmed this result. A higher number of cells, above 20 %, showed pGp9.5 expression with no significant differences between the genotypes (TNR+/+: 23.02 % ± 8.0; TNR-/-: 21.07 % ± 6.6). Comparable numbers of neurons were also found in TNC-/- and TNC+/+ cultures with 9.45 % ± 1.6 and 12.05 % ± 4.2 respectively, revealed by β-tubulin staining (ttest: $p > 0.05$; $N = 4$). MBP is expressed by all differentiated cells of the oligodendrocytic lineage. Staining with antibodies directed against MBP showed a typical cytoplasmic expression around the small and oval nuclei and along processes in a few patches of the culture. At most 1.6 % of cells were MBP positive with similar numbers comparing the genotypes. 0.78 % (± 0.2) TNR+/+ and 0.58 % (± 0.3) TNR-/- cells expressed MBP and in TNC+/+ and TNC-/- cultures 1.57 % (± 0.6) and 0.41 % (± 0.1) were found to be MBP positive, respectively. In both cases the differences did not meet significant values (ttest TNR: $p > 0.05$; $N = 4$; TNC: $p > 0.05$; $N = 4$).

As a first step in analyzing a possible role of TNs on neural stem cell differentiation we investigated the effects of TNR and TNC deficiency on differentiating dissociated cell cultures. TN deficiency did not significantly alter the number of differentiated neurons

or oligodendrocytes but a markedly higher number of TNC negative cells acquired an astrocytic cell fate.

3.1.3 Effects of TNC deficiency on proliferation of neural stem cells

TNC is an interaction partner of the EGFR (Swindle et al., 2001) and one of the EGFR activating factors, the growth factor EGF (in addition to bFGF) was added to the medium to maintain neural stem cells in an undifferentiated proliferative state. It is conceivable that the lack of TNC modulated EGFR signalling or in other means influenced the overall proliferation of the cells which may account for the detected effects on astrocytic cell numbers. To investigate whether TNC deficiency influenced the growth factor response of the neural stem cells, adherent cultures were maintained for two hours in BrdU after growing for two days in the presence of growth factors. Cells that underwent a mitotic cycle during this period could be later on detected by BrdU incorporation. 22.68 % (± 4.2) of cells in the TNC+/+ culture and 25.79 % (± 2.2) of TNC deficient cells appeared to be BrdU positive, showing no apparent difference (ttest: $p > 0.05$; $N = 3$) (Fig. 10). Thus TNC deficiency had no effect on the overall proliferation of the neural stem cell culture.

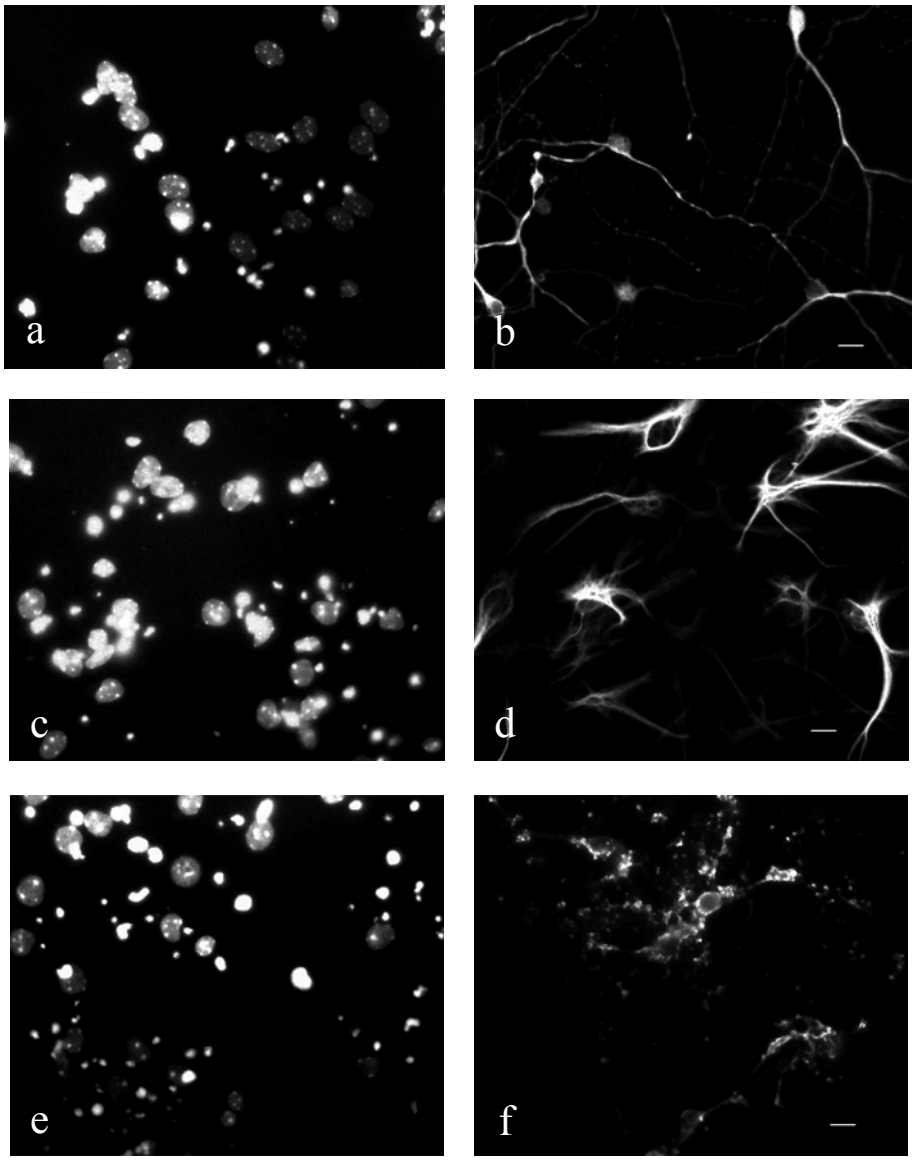


Fig. 9 Representative examples of cell type specific antibody stainings of differentiated neural stem cells
Eight days after the induction of differentiation of adherent dissociated neural stem cells the neural cell types were identified by using cell type specific markers. Left panel: staining with bis-benzimid. Right panel: specific markers. Neurons were identified by β-tubulin **(b)**, astrocytes by GFAP **(d)** and oligodendrocytes by MBP **(e)**. All markers showed typical stainings. Note the long neuronal processes stained by β-tubulin, the stained astrocytic fibrillary structures and the small oligodendrocyte somata surrounded by MBP positivity with patchy stained processes. Total cell numbers were achieved by counting bis-benzimid positive nuclei of the referring sample. Scale bars: 10 μm, valid also for the respective left picture.

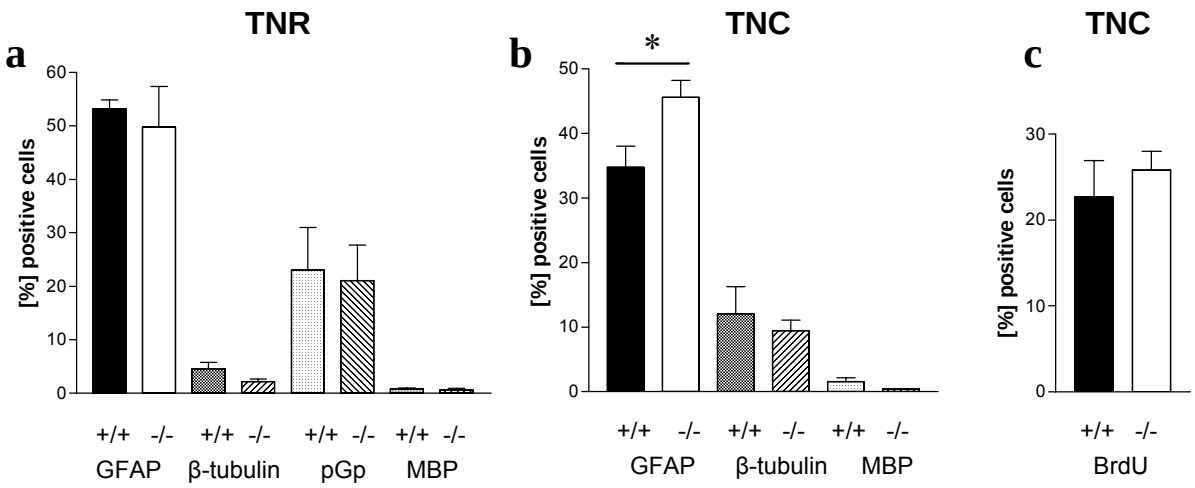


Fig. 10 Differentiation and proliferation of TN deficient neural stem cells
Numbers of astrocytes (GFAP), neurons (β-tubulin and pGp) and oligodendrocytes (MBP) 8 days after growth factor withdrawal are shown as quantified percentages of the total cell numbers for **a)** TNR - and **b)** TNC deficient neural stem cells and their wild-type littermate controls. Note the increased numbers of astrocytes in the absence of TNC. **c)** Percentage of TNC-/- and TNC+/+ cells that incorporated BrdU after an incubation period of 2 h. Results represent mean + s.e.m. of at least three independent experiments; * $p < 0.05$, using Student's t-test.

3.1.4. Establishment of an organotypic culture system co-culturing hippocampal slices with neural stem cells

TN deficiency altered the number of astrocytes in dissociated cultures of neural stem cells. Although these cultures are helpful for gaining clues for possible implications, the interpretation of the results is limited due to the great difference to the *in vivo* situation including the lack of numerous in vivo present interaction partners, factors and established cell connections. To learn about the ability to influence neural stem cells in a more physiological context we chose to investigate the behaviour of neural stem cells in organotypic hippocampal cultures. Such cultures resemble the *in vivo* environment and the situation of cells that are added to cultured slices is comparable to stem cells that are injected into different brain regions in order to investigate their potential in therapeutic use. In addition to the analysis of differentiation these cultures allow the investigation of characteristics such as invasive and migratory properties and neurite

growth that we were in particularly interested in. We began the establishment of the culture system by culturing rat hippocampus because mouse tissue has been found to be in particularly sensitive and culturing conditions are very difficult to work out. Rat hippocampal cultures could be maintained up to four weeks with a preservation of the typical cytoarchitecture. To allow the culture to settle and to reduce variations due to different stages of cell activity such as glia scar formation on the cut surface of the slice the neural stem cells were applied only after a culture period of ten days. The neural stem cells were prepared from mice that expressed the enhanced green fluorescent protein (GFP+) in all somatic cells, in order to allow the detection of the applied cells on and in the slice. Approximately 0.1 µl to 0.2 µl of a dense cell solution ($5\text{-}10_x10^4$ cells) were distributed onto the surface of the slice. After ten days a number of cells had invaded the slice and could be found within neuronal and glial areas and along fibre-tracts. The majority of cells remained on top of the slice, however, often intermingled between glial cells that built the upper closure of the slice. Many of the cells that invaded the slice and cells sitting on the upper site sent processes into the slice tissue coming in close proximity to other xenic and slice endogen cells. In all regions cells positive for the neuronal markers β-tubulin or NeuN and astrocytic proteins like GFAP or the cytoplasmic S100 (that is expressed by fibrillary and additionally protoplasmic astrocytes) were found (Fig. 13). A few cells displayed expression of markers for mature inhibitory cells such as parvalbumin or calbindin (Fig. 12+13). Parvalbumin expression in dissociated cultures of neural stem cells stained a very small number of cells almost indistinguishable from background level whereas the parvalbumin positive cells in organotypic cultures showed an intense labelling under identical analysis parameters indicating that conditions existing in the slice culture promoted the maturation of neurons (Fig. 12).

To be able to take advantage of mutant mouse lines to investigate the effects of a TN deficient environment on neural stem cell behaviour we successfully aimed to establish mouse organotypic co-cultures with neural stem cells. Mouse slice cultures showed a well preserved tissue architecture up to three weeks (Fig. 11). Due to an in average one week shorter survival time of mouse cultures, after application of neural stem cells to the slices, cultures were maintained only six more days. The behaviour of the neural stem cells during this period appeared very similar to what we observed on rat cultures.

A number of cells had migrated and sent processes into the slice. NeuN and S100 staining revealed that neurons and astrocytes developed on top and within the slice (Fig. 14).

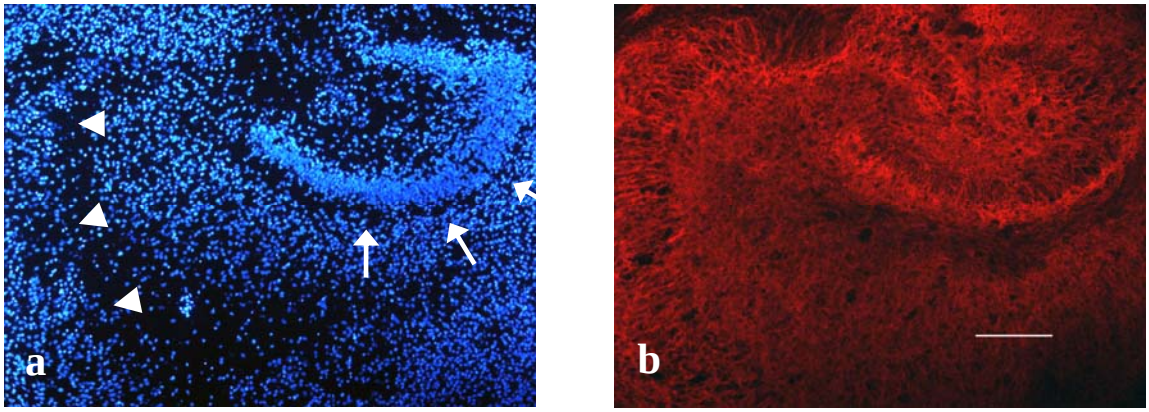


Fig. 11 Mouse hippocampal organotypic slice culture
A horizontal 10 µm section of a mouse slice at the end of a 14 day culture period shows in **a)** cell nuclei revealed by bis-benzimide staining and in **b)** the according neuronal staining with β-tubulin. Cell nuclei of the dentate gyrus (arrows) and *cornu ammonis* (arrowheads) are visible. The β-tubulin staining illustrates the preserved hippocampal tissue structure. Scale bar: 50 µm, valid for **a+b**.

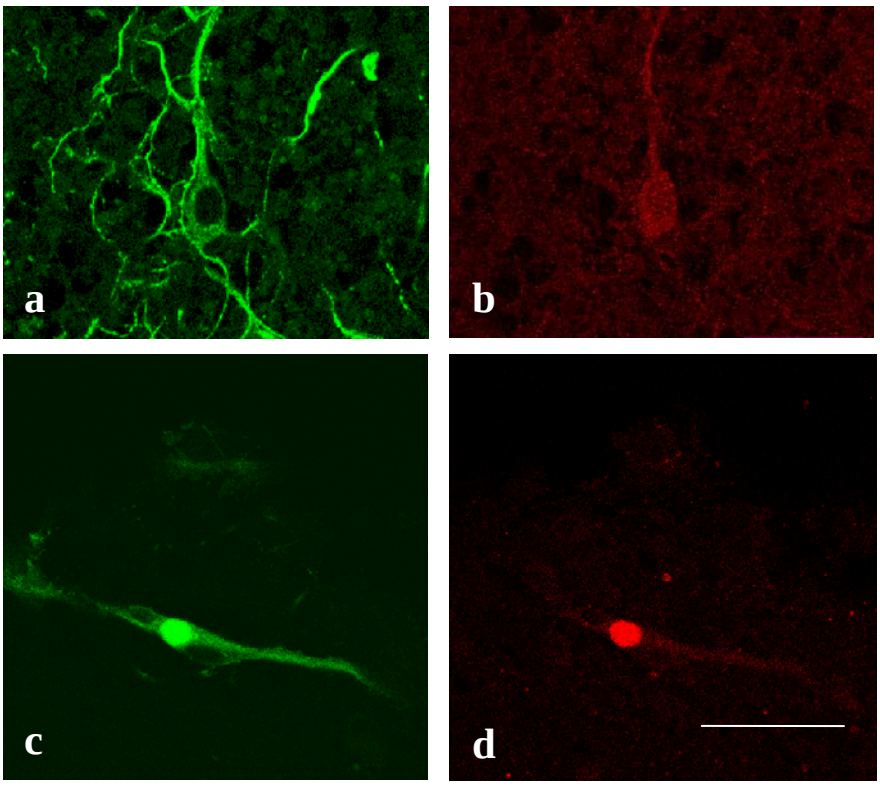


Fig. 12 Enhanced maturation of parvalbumin positive neurons on organotypic slice cultures
One GFP+ cell in a dissociated **(a+b)** and an organotypic slice culture **(c+d)** is shown that stains positively for parvalbumin, a marker for a group of inhibitory interneurons, after 8 days of differentiation of neural stem cells. Parvalbumin (right panel) signals of both cultures are detected with equal amplification and laser intensity on a confocal laserscanning microscope. The picture of the organotypic culture is taken from a horizontal 10 µm thick section of a slice. Note, the parvalbumin signal in dissociated cultures is close to background, no cell with a similar intensity as in **d)** was observed in these cultures. Scale bar: 40 µm, valid for **a-d**.

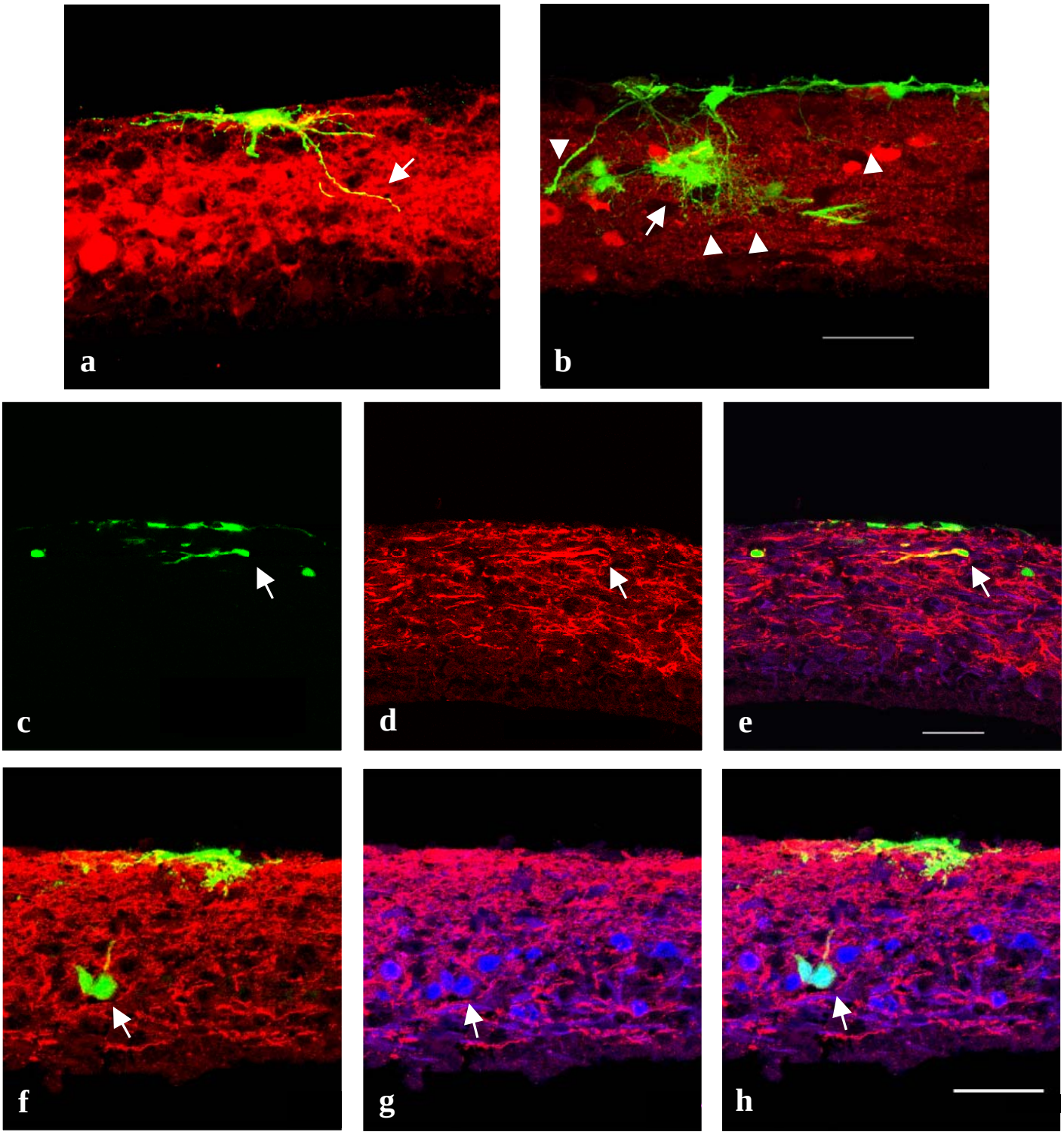


Fig. 13 Neural stem cells on / in rat hippocampal organotypic cultures
Pictures of vertical sections of rat hippocampal slices are shown. Ten days after application of GFP+ neural stem cells to hippocampal slices the cells sent processes and migrated into the slice tissue **(a+b)**. A cell that migrated into the slice tissue has differentiated neuronally, as shown by β-tubulin staining (arrows in **c-e** point to the same cell, **(e)** merged picture). Calbindin staining reveals two interneurons that integrated into the granule cell layer of the dentate gyrus (arrows in **f-h** point to the same cell, **(h)** merged picture). Note that processes of several cells get in close contact to slice endogen cells and fibers (arrowheads). Green: GFP, red in **(a+b)**: PgP9.5, red in **(d-h)**: β-tubulin, blue: calbindin. All pictures are taken with a laser scanning microscope from 20 µm vertical sections of organotypic slices. Scale bars: 40 µm, valid for each row.

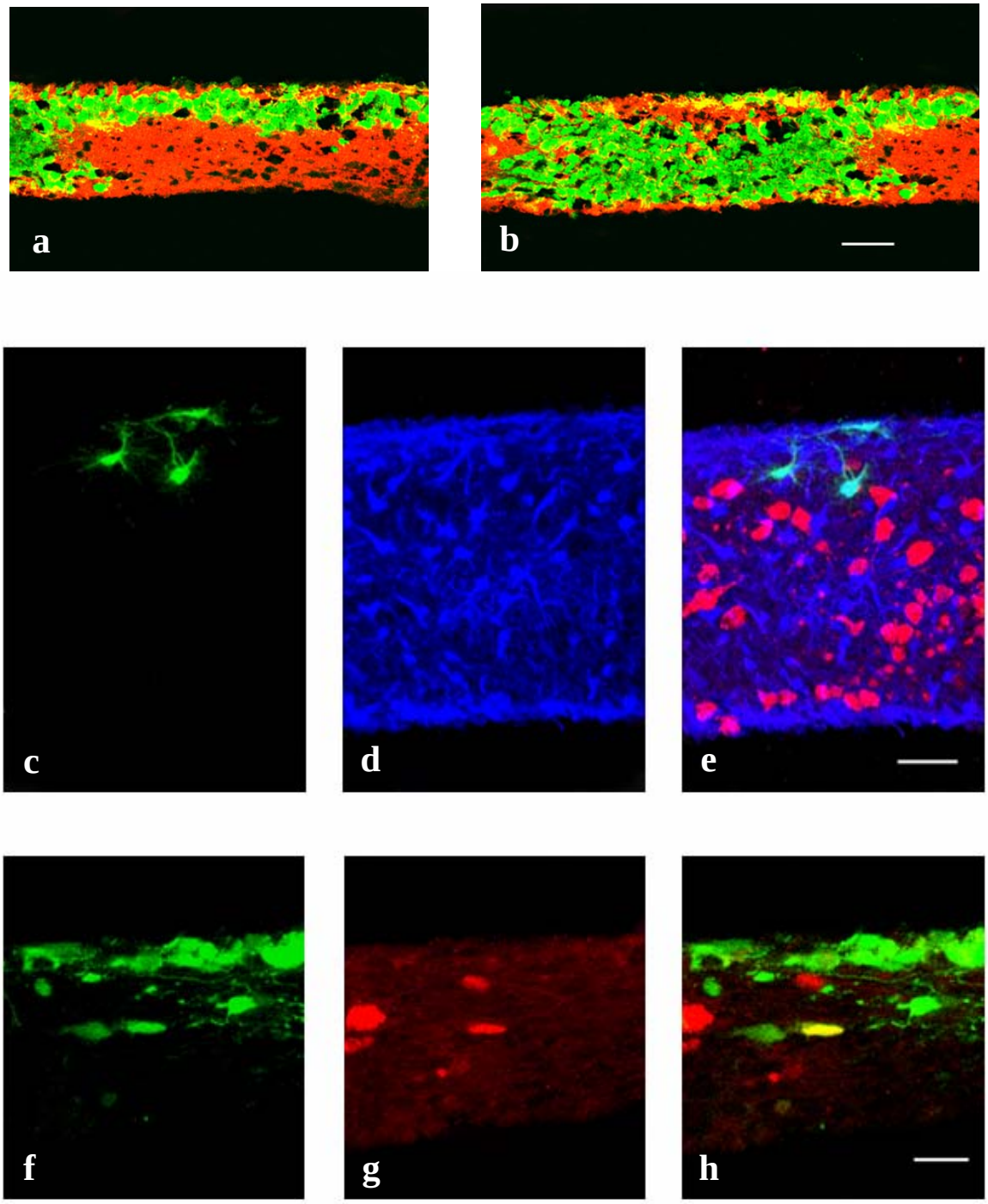


Fig. 14 TN-C expression and neural stem cells on / in mouse organotypic slices
TNC is detectable in organotypic slices around neuronal somata and regions devoid of neuronal cell bodies **(a+b)**. Neuronal somata are stained with NeuN (green in **a+b**), TNC with the polyclonal antibody KAF9 (red in **a+b**). Note that the sections also illustrate the preservation of tissue structure. **c-e)** and **f-h)** respectively show stainings of the same region. Left panel: GFP, middle: cell type specific marker and right panel: merged picture. Applied GFP+ neural stem cells have differentiated into astrocytes, revealed by S100 staining (blue) and neurons, revealed by NeuN staining (red in **c-h**) after a culture period of 6 days. All pictures are taken with a laser scanning microscope from 20 µm vertical sections of organotypic slices. Scale bars: 40 µm, for **(a+b)**, 40 µm, for **(c-e)**, 20 µm, for **(f-h)**.

3.1.5. Effects of TN deficiency on neural stem cell behaviour in organotypic cultures

The hippocampus in the adult and all the more in the developing brain is a region of TNC expression. Concomitant with this we detected TNC expression in hippocampal organotypic slices (Fig. 14 a,b). We were interested in the question whether the TNC positive environment in the hippocampus is of importance for the behaviour of neural stem cells. The opportunity to use mouse tissue for organotypic culturing allowed us to address this question and to change the environment for the neural stem cells into a TNC deficient one. Organotypic cultures were prepared from TNC+/+ and TNC-/- mice. For later detection the neural stem cells were transfected with a GFP expression vector prior to application. Six days after application of neural stem cells onto the slice culture the number of cells that invaded the slice and their maturation was determined (Fig. 15). When TNC wild-type neural stem cells were applied to TNC-/- or wild-type slices similar numbers of cells invaded the slice: 35.13 % (± 3.8) and 33.55 % (± 3.8), respectively (ttest: $p > 0.05$; $N = 6$). Staining with an antibody against S100 revealed that as observed for dissociated cells in culture the majority, though smaller percentages of matured cells differentiated into astrocytes. But in this case the numbers were independent of the genotype. 9.29 % (± 6.8) of the cells on top of TNC+/+ and 13.56 % (± 5.9) on TNC-/- slices showed S100 expression (ttest: $p > 0.05$; $N = 6$). To identify cells that differentiated into neurons we used NeuN staining. No significant differences were seen between the percentage of NeuN positive cells on TNC-/- slices compared to the wild-type slices (3.40 % ± 1.4 and 4.56 % ± 1.9, respectively; ttest: $p > 0.05$; $N = 6$). As we have stated before neural stem cells show an endogen TNC expression. To rule out the possibility that this expression obscures the deficiency of the slice we additionally compared TNC-/- neural stem cells on TNC-/- slices to TNC+/+ stem cells on TNC+/+ slices (Fig. 15b). The percentage of cells that differentiated into neurons or astrocytes showed no significant differences if TNC-/- cells applied to TNC-/- slices were compared to TNC+/+ cells on TNC+/+ cultures. Under total TNC deficient conditions 13.25 % (± 3.7) of cells were S100 positive compared to 28.33 % (± 10.0) in the wild-type situation. 1.75 % (± 1.3) TNC-/- cells expressed NeuN on TNC deficient

slices whereas among the TNC+/+ cells on wild-type slices no neuron was found. None of these differences reached statistical significance (ttest: $p > 0.05$; $N = 3$). What we could observe was a decreased number of invaded cells in case of total TNC deficiency. 17.53 % (± 4.5) of wild-type cells had invaded the wild-type slice after six days and were found within the deeper layers of the slice tissue. When TNC was missing this number decreased significantly to 4.25 % (± 2.9) (ttest: $p = 0.048$; $N = 3$).

Investigating the effects of TNC deficiency on neural stem cells in a complex *in vivo*-like culture system we could not support the finding from dissociated cultures in which TNC deficiency led to increased astrocytic numbers. However, when in addition to the environment the applied cells were devoid of TNC a reduced number of cells was found within the slice tissue pointing to an altered migration.

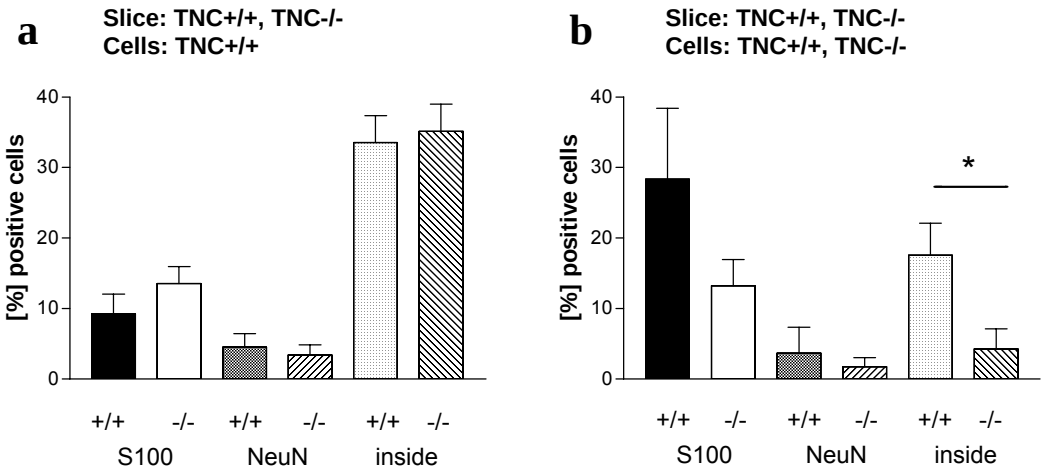


Fig. 15 Impact of TNC deficiency on differentiation and migration of neural stem cells on organotypic cultures

Relative numbers of astrocytes (S100), neurons (NeuN) on top of slices and cells which were found within the slice tissue (inside) are shown. In **a)** wild-type neural stem cells were applied to TNC+/+ and TNC-/- slices. In **b)** TNC+/+ neural stem cells were applied to TNC+/+ slices and TNC-/- cells to TNC-/- slices. Values are percentages of the total cell numbers. Fewer numbers of TNC deficient cells were found within TNC deficient tissue. Results represent means + s.e.m. of at least three experiments, * $p < 0.05$, using Student's t-test.

3.1.6. Can TN expression beneficially influence neural stem cells?

Several lines of evidence point to a supportive role of TNs for migration and neurite outgrowth (see introduction). We found that TNC deficiency led to a reduced number of cells that could be detected within the tissue of an organotypic slice culture suggesting a role in migration or cell motility. If we can drive constitutive expression of TNC and TNR in neural stem cells we may be able to beneficially influence their behaviour e.g. promote their migration, their neuritic growth or direct their fate of differentiation.

3.1.6.1. Cloning of expression constructs

The longest form of TNC is highly expressed in the early development but in adulthood, remarkably, mostly restricted to areas of ongoing plasticity suggesting an implication in plastic changes of nervous tissue, by effecting synaptic changes, influencing neuritic growth or cell migration. Furthermore a neurite outgrowth promoting function of TNC has been attributed to the alternatively spliced region (Meiners et al. 1999). Thus, it is highly suggestive that the long splice variant could promote a somewhat dynamic cell state and be responsible for the migratory deficit we observed under TNC lacking conditions. For this reason we have chosen to express the full length form of the longest splice variant of TNC and TNR to investigate the effects of their constitutive activation on neural stem cells. TNR and TNC expression vectors were generated by insertion of the long splice variants of TNR and TNC into the pcDNA3 expression vector. In consequence transcription was driven by the CMV promoter that drives expression at high levels in most mammalian cell types (Invitrogen). The resulting constructs were named pcDNA3-TNR and pcDNA3-TNC respectively. The pcDNA3-TNR plasmid was generated by inserting a 6.2 kb SalI rat TNR full-length cDNA fragment into the XhoI site of pcDNA3 (Dr. M. Kutsche). Correct orientation of the insert could be validated by EcoRI digestion which results in fragments of 0.9, 2.3, 3.0, and 5.4 kb. To generate the pcDNA3-TNC expression vector, a 6.8 kb EcoRI-SalI cDNA fragment encoding the long mouse TNC splice variant was inserted into the EcoRI-XhoI site of the multiple cloning site of the pcDNA3 vector resulting in a 12.3 kb vector. The extinction of the XhoI site in the vector following ligation was utilized to eliminate residual empty pcDNA3 plasmids by XhoI digestion after ligation. Clones with correctly inserted

cDNA were identified by BamHI restriction which resulted in three fragments of 2.3, 3.1 and 6.8 kb (Fig. 16) and was additionally verified by partial sequence analysis.
Prior to a functional investigation of the constitutive TN expression, the plasmids had to be tested upon their effectiveness regarding protein expression.

3.1.6.2. TNR and TNC are expressed by transfected cells

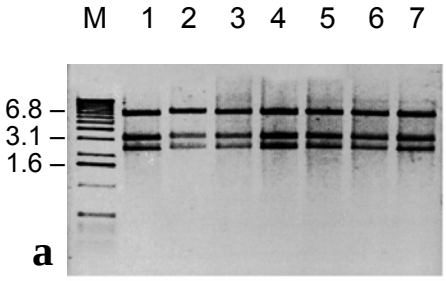
BHK cells did not show any endogenous TNR or TNC expression thus proved to be a suitable cell line to analyze the usefulness of the constructed expression vectors. 48 h following co-transfection of the cells with pcDNA3-TNR or pcDNA3-TNC in addition to a GFP expression vector for the control of transfection, TNR and TNC expression could be immunohistochemically detected in the corresponding GFP+ cells (Fig. 16). In contrast control cells that were transfected with the empty pcDNA3 vector showing GFP expression were immunonegative for TNR as well as TNC. Hence the constructed expression vectors efficiently drive TNR respectively TNC protein expression.

3.1.6.3. TN expression can be induced in neural stem cells

With the aim to study the effects of constitutive TN expression on neural stem cells we also analyzed whether the expression constructs pcDNA3-TNR and pcDNA3-TNC will efficiently drive expression in neural stem cells. Dissociated neural stem cells were transfected with the expression plasmids pcDNA3-TNR and pcDNA3-TNC and protein expression was tested 48 h later by immunohistochemistry and Western blotting (Fig. 17). For immunohistochemical detection as a transfection control cells were cotransfected with the GFP expression vector. Transfection of the cells with pcDNA3-TNR led to efficient expression and secretion of the protein. Western blotting of the culture supernatant and subsequent secondary antibody detection of the TNR protein showed a distinct band at approximately 180 kD for the culture supernatant obtained from neural stem cells transfected with the pcDNA3-TNR expression vector and no band for the control cells that were transfected with the empty pcDNA3 vector. The pcDNA3-TNC expression plasmid was tested in a similar way. The endogenous expression of TNC by neural stem cells required standardization of the amount of protein, for which we used the extracellular cleaved form of APP. If the values of

densitometric measurements of the high molecular weight form of TNC at around 220 kD were standardized to the amount of APP the transfection of neural stem cells with the expression vector pcDNA3-TNC led to a doubling of TNC protein in the supernatant of cultured cells after 24 h (2.2 ± 0.08; ttest $p < 0.001$; $N = 7$). In addition to the bands at a molecular weight of 220 kD higher molecular bands appeared in probes of transfected cells. We suppose that these are TNC molecules that associated with each other due to high concentrations in the probe indicating that the amount of additionally secreted TNC after pcDNA3-TNC transfection into the medium was even above 100 %. Immunohistochemical staining with a polyclonal antibody raised against TNC of adherent transfected cells evidently cannot show the over-expression but intuitively the result underscored the biochemical finding (Fig. 17).

The biochemical analysis of cell supernatant could validate that both vectors, pcDN3-TNR and pcDNA3-TNC, efficiently drive expression of protein in neural stem cells, allowing us to move on to investigate functional effects of a constitutive TN expression on neural stem cells.



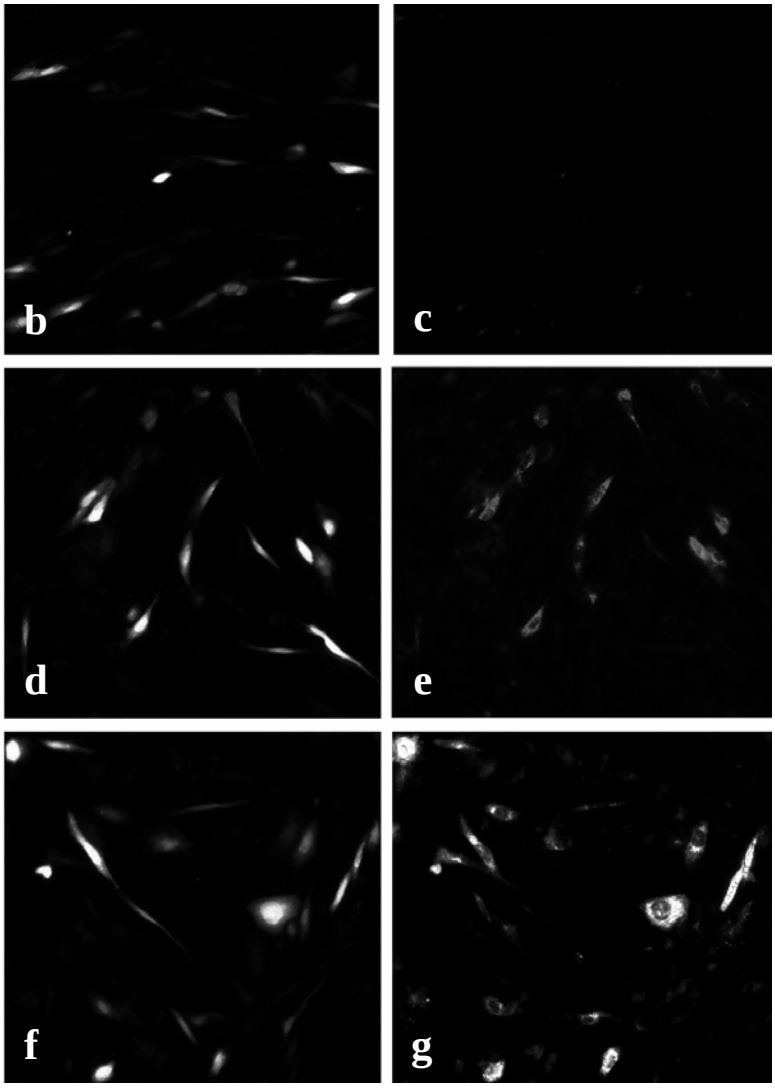


Fig. 16 Analysis of the expression vectors pcDNA3-TNR and pcDNA3-TNR

a) Vectorcloning pcDNA3-TNC. BamHI restriction of pcDNA3-TNC reveals 7 clones that show the 3 fragments at 2.3, 3.1 and 6.8 kb, expected with correct insertion of the TNC fragment. M: 1 kb DNA ladder, on the left margin the apparent size is indicated in kb.

b-g) Test of protein expression in BHK cells.

BHK cells were co-transfected with a GFP expression vector and – as a control with the empty pcDNA3 vector **(b,c)**, – pcDNA3-TNR **(d,e)** or – pcDNA3-TNC **(f,g).** 48 h following transfection protein expression can be detected using the monoclonal antibody clone 619 and the polyclonal KAF9(2) antibody directed against TNR and TNC, respectively. Left panel: GFP+ cells. Right panel: specific antibody staining. In the control cells no TNR and TNC immunoreactivity is detected **(c),** pcDNA3-TNR transfected cells show TNR expression **(e)** and pcDNA3-TNC transfected cells express TNC **(g).**

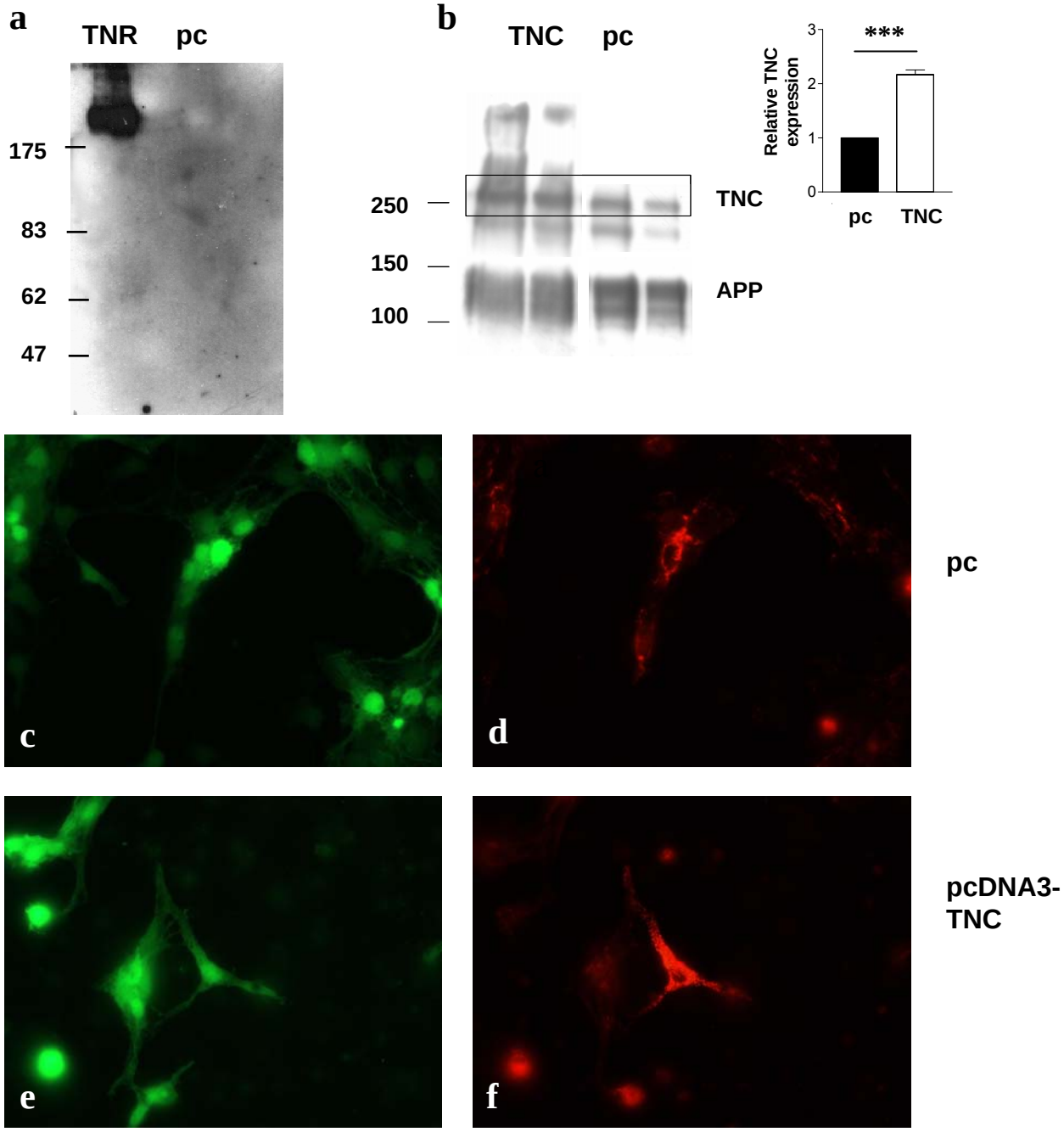


Fig. 17 Control of protein expression in neural stem cells induced by constitutively active TN expression vectors
48 h after transfection of neural stem cells with the expression constructs pcDNA3-TNR and pcDNA3-TNC, TNR and TNC protein is detected in cell supernatant by Western blotting using the monoclonal antibody clone 596 and polyclonal KAF9(2) respectively **(a+b)**. A strong TNR positive band is apparent in the supernatant from pcDNA3-TNR (TNR) transfected stem cells at approximately 180 kD but not from cells transfected with the empty vector (pc) **(a)**. The amount of TNC protein was standardized to the extracellular cleaved part of APP because of the cell endogen expression of TNC. APP bands are apparent at around 100 kD and 120 kD. The applicable 220 kD TNC band is framed. Molecular weight markers are indicated at the left margins in kD. **b)** Densitometric measurements of according bands show an expression of TNC by pcDNA3-TNC (TNC) transfected cells that is twice as high as with cells transfected with the empty control vector (pc) (graph in **b**).
TNC over-expression after pcDNA3-TNC transfection becomes also apparent in immunohistochemical stainings **(c-f)**. pcDNA3 transfected cells **(e+f)** show a brighter staining than control transfected cells **(c+d)**. green: GFP, red: TNC staining using the polyclonal antibody KAF9 (2). Results in **(b)** represent means + s.e.m. of seven experiments after normalization to the control value. *** $p < 0.001$, using Student's t-test

3.1.7. Effects of constitutive TNR and TNC expression on neural stem cells

The constructed expression vectors could be used now to drive constitutive expression of TNR and TNC in neural stem cells in order to investigate whether neural stem cells can possibly beneficially be influenced by TN expression e.g. by promoting migration, neuritic growth or by modulating their fate of differentiation.

Dissociated neural stem cells were co-transfected with the respective TN- and the GFP-expression vector and applied onto organotypic cultures. To control successful transfection a part of the cell suspension was plated onto PLL-coated coverslips and grown for 24 h under the influence of growth factors. The supernatant was tested upon expression by Western blotting and subsequent secondary antibody detection.

TNR expression by neural stem cells did not change the number of cells that could be found within the slice ten days after application of the cells.

Different regions of the hippocampus are known to express different sets of molecules that may influence the behaviour of the added cells in a specific manner. Possibly TNR expression exhibited some effects in one part of the slice but those effects were covered by looking at the total cell population. For this reason we discriminated between the dentate gyrus and the remaining part of the hippocampal formation (from hereon called CA region) by their differential calbindin expression. Granular cells of the dentate gyrus exhibited strong calbindin expression whereas within the *cornu ammonis* only interneurons scattered throughout the layers were positive for calbindin (Fig. 18).

By combining the staining against calbindin to determine the hippocampal region with the neuronal marker β-tubulin we investigated migration, neuronal differentiation and neurite growth of the cells dependent on their location (Fig. 19+20). The majority of cells could be found within the slice tissue of the dentate gyrus with no apparent changes due to TNR expression: 30.28 % ± 6.6 of cells expressing TNR and 27.41 % ± 6.0 of cells of the control group invaded the dentate gyrus. Cell numbers that migrated into the CA region did not show significant differences between TNR transfected cells (11.38 % ± 4.2; N = 6) compared to cells transfected with the control plasmid (21.35 % ± 3.3; N = 5; ttest: $p > 0.05$). Similar numbers of cells expressing TNR compared to control cells also differentiated into neurons on top and within the

slice. 29.61 % ± 8.4 control cells and 27.56 % ± 9.1 TNR transfected cells sitting on top

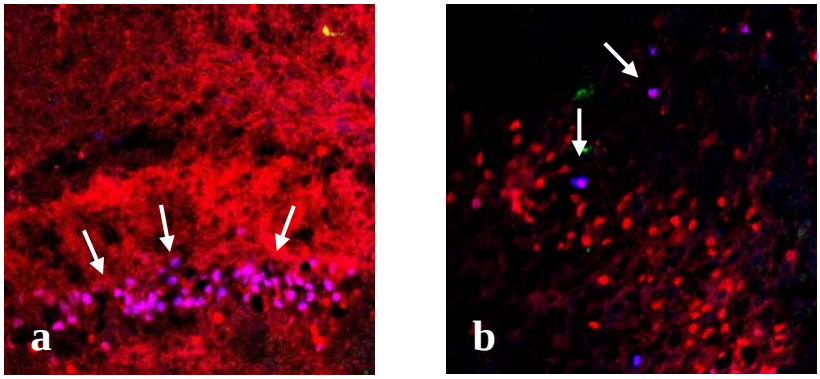


Fig. 18 Calbindin staining discriminates hippocampal regions
Shown are two different parts of one horizontal section of a rat organotypic hippocampal slice. Antibody detection of calbindin brightly stains the band of granule cells in the dentate gyrus (**a**, blue staining, violet cells, arrows). The neuronal pGp9.5 counterstain (red) leads to a violet appearance of the cells. In the *cornu ammonis* **(b)** only few scattered cells show calbindin expression (arrows). The neuronal counterstain reveals the neuronal structure and neurons in the dentate gyrus and in the *cornu ammonis*.

of the slice showed β-tubulin expression (ttest: $p > 0.05$; $N \geq 5$); within the slice the percentages were: 20.84 % ± 5.1 control cells and 18.21 % ± 3.2 TNR expressing cells (ttest: $p > 0.05$; $N \geq 5$). Furthermore, the number of neurons sitting on the surface of the slice and sending a neurite into the slice tissue was determined as an indicator for neurite growth. TNR expression did not alter neuritic growth into the slice tissue. 46.34 % ± 13.7 of neurons in the control group sent a process into the slice compared to 38.64 % ± 9.4 of TNR expressing neurons.

Unlike the result from TNC deficient cultures had suggested neural stem cells that were transfected to constitutively express TNC were not found in different numbers within the slice if compared to the cells transfected with the control plasmid. As seen for TNR expressing cells more cells migrated into the dentate gyrus than into the CA region in similar numbers comparing TNC expressing and control cells. 10.79 % ± 3.5 control cells and 12.33 % ± 4.6 TNC constitutively expressing cells were found within the slice tissue in the dentate gyrus and 6.37 % ± 0.6 and 6.93 % ± 2.0 in the CA region, control and pcDNA3-TNC transfected cells respectively (ttests: $p > 0.05$; $N = 6$). The lack of TNC had led to an increase in the number of astrocytes in dissociated cultures of neural stem cells thus we investigated the astrocytic fate in the organotypic cultures by S100

staining. The number of TNC over-expressing cells showing S100 expression was not significantly different as compared to the control cells. (Control transfected: 4.39 % ± 1.6, pcDNA3-TNC transfected: 12.41 % ± 8.6; ttest: $p > 0.05$; $N = 6$). Similarly β-tubulin staining revealed no difference between both groups. 7.09 % ± 1.1 neurons were found within the control group and 8.95 % ± 2.0 among pcDNA3-TNC transfected cells on top of the slice (ttest: $p > 0.05$; $N = 6$) . Is the percentage of neurons among the cells within the slice determined differences occur that do not reach significance. 26.27 % ± 8.4 of the control cells within the slice showed β-tubulin expression whereas this number decreased not significantly to 7.17 % ± 3.4 for the cells constitutively expressing TNC (ttest $p = 0.06$; $N = 6$). The number of neurons sending a neurite into the slice tissue was also alike: 21.62 % ± 7.5 and 20.37 % ± 8.7 control - and TNC expressing cells, respectively (ttest $p > 0.05$; $N = 6$). In addition to β-tubulin we used the marker NeuN to identify neurons. In distinction to β-tubulin that has been reported to be one of the earliest markers of neuronally committed cells, NeuN is expressed later during differentiation and is recognized as a marker for more mature neurons (Caccamo et al., 1989; Mullen et al., 1992). Concomitant with this, smaller numbers of positive cells were found in our culture with significant differences between both opposing groups. Whereas 3.25 % ± 0.8 of the control cells were found to be NeuN positive this value markedly decreased with constitutive TNC expression to 0.56 % ± 0.3 (ttest $p = 0.006$; $N = 7$; Fig. 20).

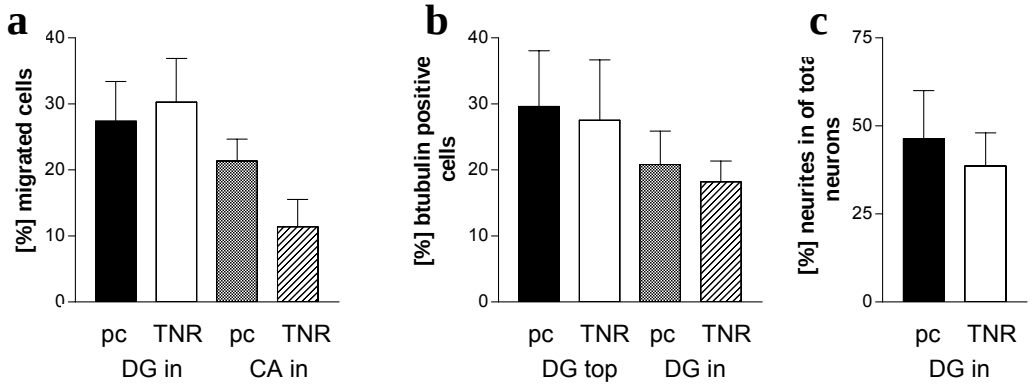


Fig. 19 Migration, neuronal differentiation and neurite growth of TNR expressing neural stem cells
Ten days after application to hippocampal slices the behaviour of neural stem cells transfected with pcDNA3-TNR (TNR) and the control (pc) cells transfected with the empty vector was analyzed. Relative numbers of cells which invaded the dentate gyrus (DG in) or the remaining part of the hippocampus (CA in) **(a)** and differentiated neuronally, revealed by β-tubulin expression on top (DG top) and within the dentate gyrus (DG in) **(b)** are shown. Independent of the transfection vector more cells invaded the dentate gyrus **(a)**. The number of neurons is comparable between TNR expressing and control cells on top and within the slice tissue of the dentate gyrus. In **(c)** percentages of neurons that sent processes into the dentate gyrus are shown. Results represent means + s.e.m. of at least five experiments. DG: dentate gyrus, CA: hippocampus without DG, pc: control

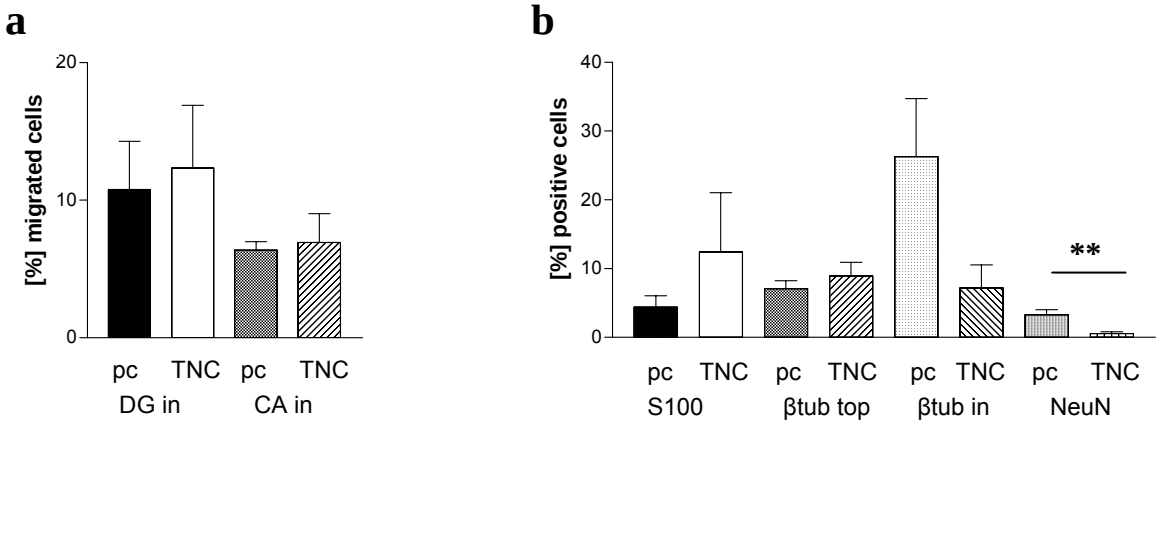


Fig. 20 Migration, differentiation and neurite growth of TNC expressing neural stem cells

Ten days after application the behaviour of neural stem cells transfected with pcDNA3-TNC (TNC) and the empty vector (pc) on hippocampal slices was analyzed. Relative numbers of cells which invaded the dentate gyrus (DG in) or the remaining part of the hippocampus (CA in) **(a)**, differentiated into astrocytes (S100) and neurons (β-tubulin and NeuN) on top (DG top) and within the dentate gyrus (DG in) **(b)** are shown. As seen before (Fig. 19) more cells invaded the dentate gyrus independent of the transfection vector **(a)**. The number of astrocytes and β-tubulin positive neurons is comparable between TNR expressing - and control cells on top and within the slice tissue of the dentate gyrus. Fewer TNC constitutive expressing cells show NeuN positivity **(b)**. In **c)** percentages of neurons that sent processes into the dentate gyrus are shown. Results represent means + s.e.m. of six experiments. ** $p < 0.01$, using Student's t-test. βtub: β-tubulin, DG: dentate gyrus, CA: hippocampus without DG, pc: control

The constitutive expression of TNs did not as we had aspirated promote the migration of applied cells into cultured slices, nor did it enhance neurite growth. The number of astrocytes and β-tubulin positive neurons did not significantly change with TN expression but the constitutive expression of TNC markedly decreased the number of mature NeuN positive neurons. Does this effect depend on other matrix molecules or factors present in the tissue environment of the organotypic culture or will we obtain a similar result in a pure dissociated stem cell culture?

3.1.8. Effects of TN-C expression in dissociated cultures

We transfected neural stem cells with the TNC expression vector and the empty control plasmid, plated the cells onto PLL-coated coverslips and induced differentiation by withdrawal of growth factors. After 8 days of differentiation the numbers of astrocytes and neurons were determined by S100 and NeuN staining (Fig. 21). After normalization to the control value of one experiment nearly the same number of astrocytes was found in TNC over-expressing cells and control transfected cells (1 ± 0.18, N=3). As expected from the comparison of parvalbumin positive neurons in dissociated and organotypic cultures that suggested a promotion of stem cell maturation on slice cultures lower numbers of NeuN positive neurons were found than were observed in organotypic cultures. And the constitutive expression of TNC led to a decrease in NeuN positive cells. Half (0.50 ± 0.1; ttest $p < 0.01$; N = 3) of the TNC expressing cells expressed the mature neuronal marker NeuN compared to the cells transfected with the empty vector, confirming the result obtained in organotypic cultures and indicating that this effect is independent of factors of the cultured hippocampal tissue.

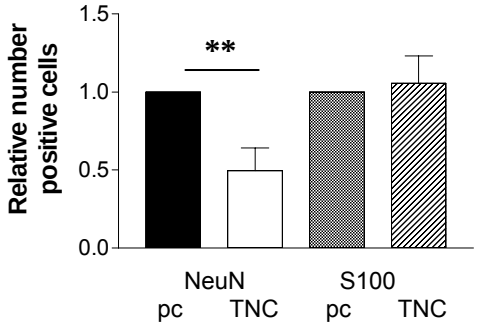


Fig. 21 Differentiation of dissociated TNC expressing cells
Constitutively TNC expressing cells show a by half reduced number of NeuN positive cells in dissociated cell cultures 8 days after growth factor withdrawal, whereas the number of astrocytes (S100) is not altered. ** $p < 0.01$, using Student's t-test

3.1.9. TNC over-expression prolongs EGFR expression

TNC is a potential binding partner for the EGFR whose expression is altered in TNC deficient mice (Swindle et al., 2001; Garcion et al., 2004). Does the constitutive expression of TNC alter EGFR expression and may such interference underlie the decreased neuronal maturation / differentiation?

To investigate this question we used dissociated cultures of neural stem cells for a biochemical approach. After transfection with the TNC expression vector and the control vector the neural stem cells were plated onto PLL-coated dishes. The level of EGFR expression was analyzed prior to the induction of differentiation and after a period of four days by Western blotting of the lyzed cells. Two experiments revealed that constitutive TNC expression prolongs or enhances the expression of the EGFR under differentiating conditions. In both experiments the level of EGFR decreased strongly with time in the control cultures. To the contrary in one experiment TNC expression lead to a twelve fold increase of the EGFR after four days of differentiation. This drastic effect was not observed in a second experiment in which still the decrease of the EGFR expression level was reduced in comparison to the control cells (Fig. 22). After four days of differentiation seven times more EGFR protein was detectable in TNC constitutive expressing cells compared to control transfected cultures after normalization to total Erk values.

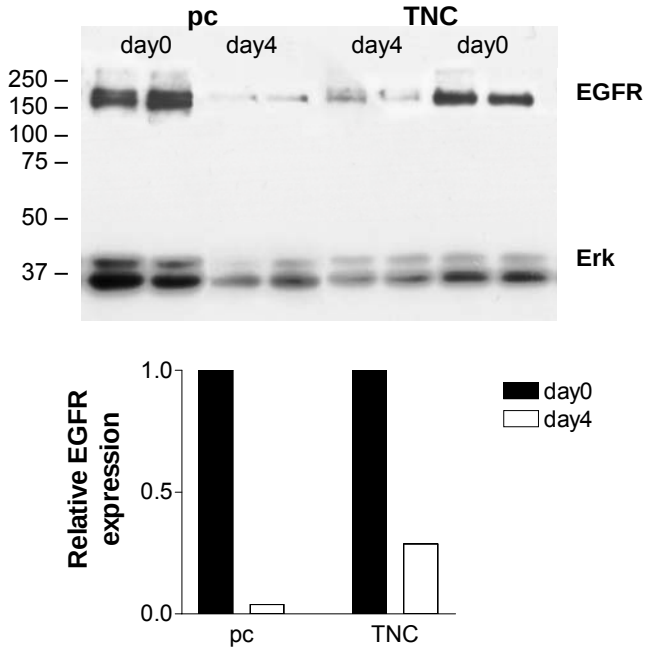


Fig. 22 Expression of the EGFR by NSCs
Prior to growth factor withdrawal and four days later the level of EGFR expression was analyzed by Western blotting. The EGFR band is detectable at around 170 kD. To standardize the number of living cells the amount of total Erk protein was used. Note for the control cells (pc) the amount of protein appears to be higher. At day 4 relatively more EGFR is present in constitutive TNC expressing cells (TNC) than in the control cells. Molecular weight markers are indicated at the left margin in kD. The graph shows the relative amount of expressed EGFR after standardization to total Erk protein and normalization. Whereas in the control transfected cells the level of EGFR decreased after four days to 4 % in TNC constitutive expressing cells it still holds 28 % of the amount of protein that was detected before the induction of differentiation.

We started to investigate a possible influence of TNC expression on EGFR signalling and found that constitutive TNC expression prolonged the expression of the EGFR by differentiating neural stem cells. Thus a modulation of EGFR expression by TNC could be involved in the observed effects such as a decreased number of mature NeuN positive neurons that appeared with constitutive TNC expression and the changed numbers of astrocytes or reduced rates of migrated cells that were observed with TNC deficiency.

3.2. Transgenic mouse

A transgenic mouse with over-expression of the TNC long-splice variant in astrocytes should help to elucidate the function of TNC in early brain development. The human GFAP promoter was utilized to drive TNC expression. This promoter was previously shown to drive a strong expression and additionally to be activated in mouse earlier than the endogen mouse GFAP promoter (Zhuo et al., 1997). Because of the earlier activation of TNC expression in these mice we hoped not only to gain further insight into functions of TNC by possible aberrations due to an excess of the molecule but in addition to find out whether the premature onset of TNC expression supports or even induces developmental processes and e.g. supports an *in vivo* function of our observed effects in culture.

The mouse full-length long splice variant of TNC was available in a vector designated pm68H14 (Dr. M. Kutsche). Due to a lack of appropriate restriction sites, in a first step this vector was modified by insertion of a polylinker encoding the restriction sites SpeI-BglII-AgeI-EcoRI into the multiple cloning site. Correct insertion was verified by detecting two bands at about 3.0 and 6.0 kb after digestion with XhoI and BglII (Fig. 23a). Secondly a BglII-AgeI fragment containing the 2.2 kb hGFAP promoter was inserted into the new BglII-AgeI restriction sites of the modified pm68H14 vector generating the final pm68H14-hGFAP-TNC vector. Correct ligation was verified by generation of two fragments of about 4.0 and 8.0 kb after digestion with XhoI and partial sequencing using the primers GFAP4 and GFAP8 (Fig. 23b; for more details see Materials and Methods).

Animals that showed integration of the DNA fragment into their genome were identified by Southern blotting of isolated genomic DNA from tail biopsies. From two blastocyte injections six positive animals derived. Fig. 23c shows an example blot that revealed two positive animals. The amount of integrated copies was not determined but

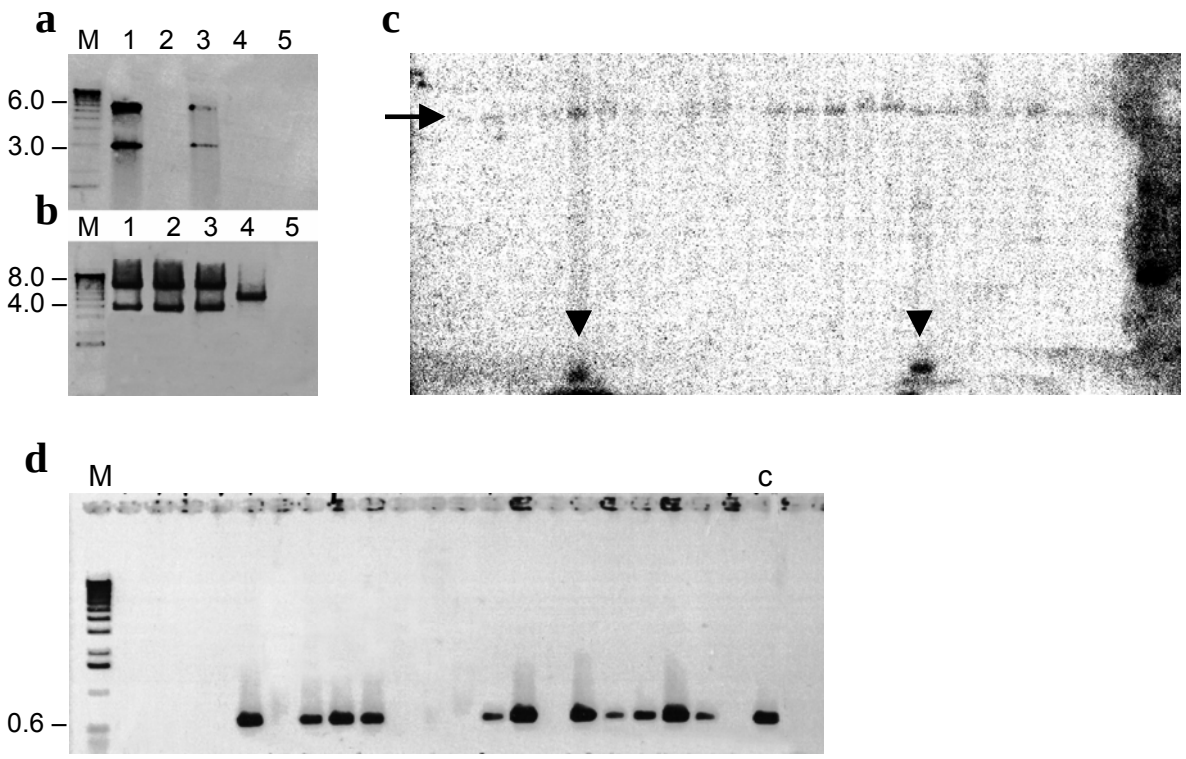


Fig. 23 Generation of the hGFAP-TNC mutant mouse
a) Vectorcloning pm68H14-hGFAP-TNC step1: Successful modification of pm68H14 by insertion of a polylinker was validated by restriction with XhoI and BglII. The gel picture shows the correct 2 bands of 3.0 and 6.0 kb for two clones in lane 1 and 3. Lane 2 and 4 are empty lanes, lane 5: water control.
b) Vectorcloning pm68H14-hGFAP-TNC step2: Correct insertion of the hGFAP promoter fragment into the modified pm68H14 was validated by restriction with XhoI. The correct bands at 4.0 and 8.0 kb can be detected for clones in lane 1, 2 and 3. The clone in lane 4 was assumingly not cut. Lane 5: water control.
c) In the shown example 23 digested genomic DNA samples from tail biopsies of potential transgenic animals were analyzed. The control bands are visible on the upper side of the blot (arrow). Arrowheads point to 2 positive bands. Note the higher intensity of the positive bands in comparison to the control bands suggesting a higher number of copies that have integrated into the genome. The picture was developed for 20 min.
d) Determination of genotypes by PCR. Gel analysis of a PCR using primers primer 1: hGAFP and primer 2:TNCn224 as performed routinely with representative DNA samples to identify genotypes. The example picture of 21 analyzed tail biopsies reveals 11 positive animals in addition to the control from an animal that was positive in Southern blot analysis.

comparing the intensities of the positive fragment band with the control bands suggested the existence of several copies.

The positive animals were used as founder animals for separate lines and taken into crossings with C57BL/6J mice. Among all litters of the following generation positive animals were found indicating that the inserted fragment(s) had entered the germ line in all cases; however, one founder animal did not produce any progeny at all. The successful transcription of the inserted fragment was analyzed on the progeny of the founder animals by quantitative real-time PCR. From the five breeding founder lines transgenic descendants of three lines showed a higher level of TNC transcript compared to the wild-type littermates in real-time PCR analysis. After standardization to the house-keeping gene HPRT the TNC expression in these three lines showed a 1.4 - , 2.5 - and 1.5 fold difference in gene expression, derived by the equation:

$$\text{Standardized transgene / Control} = 2^{\Delta Ct\ target} / 2^{\Delta Ct\ stand}$$

With ΔCt = Difference between cycle threshold, target = target gene TNC, stand = standardizing gene HPRT.
The lines with a fold - increase in TNC expression were further investigated to determine an over-expression on the protein level by Western blotting. To obtain unambiguous results here, the animals were crossed into the TNC deficient mutant mouse line which should allow an easy detection of TNC expression induced by the hGFAP promoter of the inserted DNA fragments. Immunohistochemical stainings of

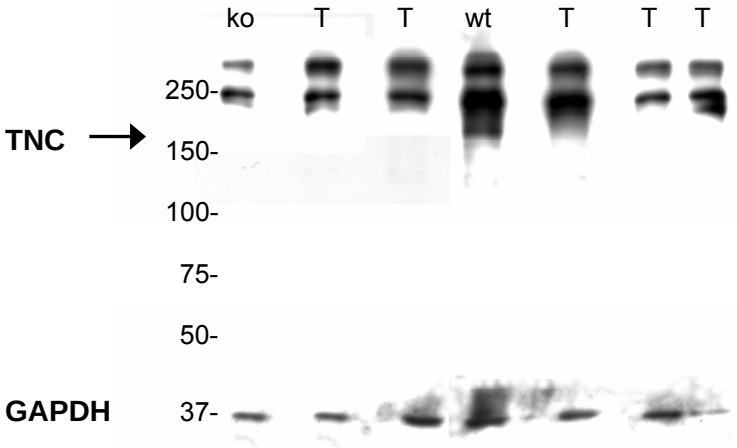


Fig. 24 Western blot of crude brain homogenate from hGFAP-TNC mice TNC expression was analyzed in crude brain extracts of 14 d old transgenic mice crossed into the TNC-/- background (T). The two upper bands represent non-specific binding of the anti-TNC polyclonal antibody pk7 as revealed by TNC-/- tissue (ko). None of the hGFAP-TNC transgenic animals showed the TNC specific band seen in the wild-type brain (wt). GAPDH was used as the internal control. Molecular weight markers are indicated at the left margin in kD.

brain sections of transgenic animals in the TNC deficient background did not reveal any TNC signals and were indistinguishable from knock-out sections, whereas sections of transgenic animals in wild-type background showed no differences in comparison to wild-type sections (Fig. 25). Western blot analysis of crude brain homogenate using wild-type, knock-out and transgenic animals in the TNC deficient background underscored the immunohistochemical finding (Fig. 24).

Although three founder lines exhibited an increased level of TNC transcript the analysis of TNC protein revealed that our aim to generate a transgenic mouse line over-expressing TNC in astrocytes was not successful.

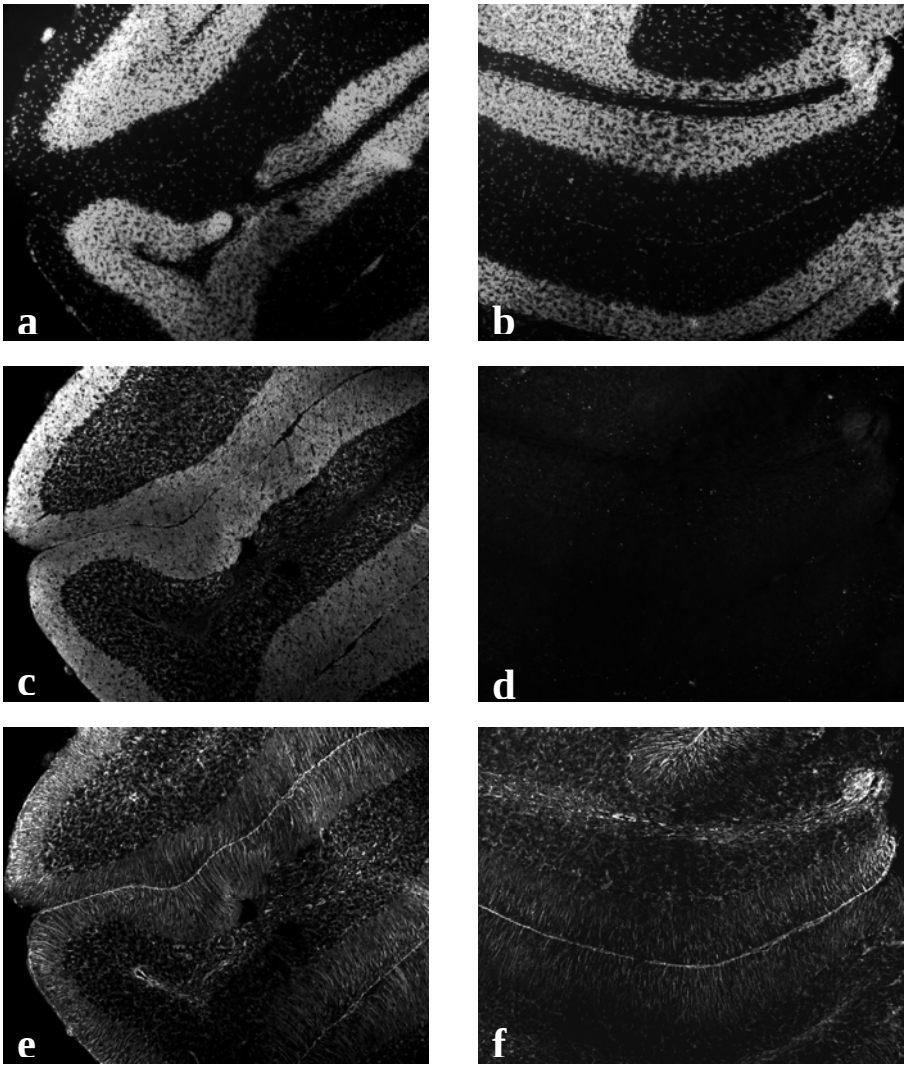


Fig. 25 Immunohistochemical analysis of hGFAP-TNC transgenic mice
TNC expression was detected on fresh frozen 10 µm sections of cerebella from 6 weeks old transgenic hGFAP-mice in the TNC-/- background (right panel) and their TNC+/+ littermates (left panel). Bis-benzimide staining **(a,b)** shows the cell layer of the cerebellum, polyclonal anti-TNC antibody KAF9(2) **(c,d)** reveals intense labelling on wild-type sections **(c)** whereas no significant immunoreactivity is detectable in the transgenic mice **(d)**. GFAP is detected at normal levels **(e,f)**.

4.0 Discussion

4.1. Do TNs influence neural stem cell development?

4.1.1. TNR and TNC can be detected in neural stem cell cultures

TNC expression by proliferating and undifferentiated striatal neural stem cells in culture corresponds to the described temporal expression in mouse brain from embryonic day ten, including the subventricular zone (Kawano et al., 1995; Garcion et al., 2004) and is reinforced by Suslov et al. (2002) who detected TNC mRNA in a number of single neurospheres. Under the light of the relatively late onset of TNR expression in mice around birth (Bartsch et al., 1993) it was not astonishing to find no TNR expression in E14 neural stem cell cultures. Its expression two days after induction of differentiation by withdrawal of growth factors is likely due to oligodendrocyte progenitors as we could not detect mature oligodendrocytes at this early stage and the protein has been found to be expressed by cultured progenitors of the oligodendrocyte-type-2-astrocyte lineage (Jung et al., 1993).

4.1.2. Impact of TN deficiency on neural stem cell differentiation *in vitro*

We investigated the roles of TNR and TNC during differentiation of neural stem cells in dissociated cultures. As both molecules are secreted and could be detected in the culture supernatant it was conceivable that the total cell population would be influenced by TNR and TNC expression. However the lack of TNR did not alter the numbers of differentiated neurons, oligodendrocytes or astrocytes. Pesheva et al. (1997) found TNR to be important in timing the differentiation of oligodendrocyte precursors *in vitro*. Concomitant with their finding of TNR to accelerate the maturation of oligodendrocyte precursors it is possible that the early apparent effects in our culture had been equalized until the day of analysis.

The relative number of oligodendrocytes in TNC deficient and wild-type cultures was also comparable. A reduced rate of oligo-precursor proliferation as described in the TNC deficient mouse would have altered the number of oligodendrocytes in the TNC deficient culture. Garcion et al. (2001) could show that this effect of TNC mainly depended on TNC enhancing the responsiveness of oligo-precursor cells to the mitogen PDGF. Type-1-astrocytes have been described as the major source of PDGF (Pringle et al. 1989) and may produce a basic level in the culture medium, possibly the concentrations were too low to be effective. This was not measured. In addition differences in cell death could have compensated for reduced proliferation rates, as was detected in the TNC-/- mouse (Garcion et al., 2001).

TNC influenced the number of astrocytes

Whereas the number of oligodendrocytes and neurons was not altered in TN deficient cultures the number of astrocytes was increased under TNC deficient conditions. Could differences in the proliferation of cells account for this effect? We have investigated the proliferation rate of undifferentiated cells and did not observe differences between the genotypes. Thus the overall proliferation was not altered, yet we cannot exclude differences in subsets of stem cells in the starting culture with different probabilities to generate astrocytes. Differences in neural stem cell properties have been found in the TNC deficient mouse in which more bFGF responsive cells were found (Garcion et al., 2004). We cultured the cells with addition of EGF and bFGF which may have led to a proportionally greater growth of bFGF responsive stem cells in the TNC deficient

culture compared to the wild-type culture, however bFGF responsive stem cells have been reported to more likely differentiate into neurons (Kilpatrick and Bartlett, 1995) thus not providing an explanation for the observed increase in numbers of astrocytes in case of TNC deficiency. More likely is one of three further possible explanations. First TNC could negatively control the differentiation of astrocytes here again it may change the pace of maturation or the cell fate. Secondly it may have an influence on the proliferation of astrocytes or thirdly on cell death. An instructive cue leading to astrocytic cell fate would go in expense of the number of oligodendrocytes and neurons. The numbers of TNC-/- neurons and oligodendrocytes were slightly reduced compared to the wild-type situation but the difference (approximately 4 %) could not completely account for the 10 % more astrocytes in culture. This speaks for at least an involvement of an accelerated maturation and/or enhanced proliferation of astrocytes or their precursor cells under TNC deficient conditions. Both would go partly at the expense of the group of nestin positive undifferentiated cells that make up about 50 % in our culture after eight days of differentiation. Thirdly different rates of cell death could have lead to the shifted percentages of cell numbers. Investigations of proliferation, cell death, the number of nestin positive cells and clonal analysis could clarify this issue. Hints for a role of TNC in astrocyte proliferation came from Steindler et al. (1995), who observed more astrocytes to be associated with wounds of TNC-/- mice but did not analyze this issue further. This explanation is further supported by a recent report that found TNC to reduce the proliferation rate of human adult astrocytes *in vitro* (Holley et al., 2005). If so, TNC may be an interesting candidate helping to reduce glia scarring which is still thought to inhibit the process of regeneration. Findings of Garcion et al. (2004) who report enhanced neurogenesis in connection to no changes in the number of glia when TNC deficient neural stem cells differentiated *in vitro* are contradictory to our results. However, methodological differences could contribute to this: Garcion et al. investigated whole neurospheres that adhered to the substrate and only cells that migrated into the marginal zone were included into the analysis which restricted the analyzed cells to those showing migratory potential, whereas we plated dissociated single cells thus evaluating the total cell pool. Secondly the experiments were performed with a different strain of TNC-/- mice that was found to show residual

truncated protein expression (Mitrovic and Schachner, 1995), which could have resulted in a misleading outcome.

4.1.3. Organotypic cultures are a useful model system to analyze neural stem cell behaviour

To the best of our knowledge this is the first time that neural stem cells have been co-cultured with hippocampal organotypic cultures. Benninger et al. (2003) used a similar system to investigate the behaviour of ES cell derived neural precursor cells and we established the culture according to their protocol and experiences. We observed replicable numbers of cells that adhered to the slices, invaded them and showed several characteristics of differentiation such as neuritogenesis and expression of cell type specific markers. Thus we concluded that the established co-culture method is a useful model system for analyzing migration and differentiation events of neural stem cells in a more *in vivo*-like situation than cultures of isolated dissociated single cells. Furthermore, the slice culture conditions resemble the situation stem cells are confronted with after injection into areas of the brain as done for therapeutic approaches. Thus the potential of a candidate protein, whose beneficial influence on stem cells shall be determined, can readily be examined in such cultures.

Some general aspects attracted attention by examining the co-culture such as a far smaller number of differentiated astrocytes on the organotypic culture in comparison to the *in vitro* culture using dissociated cells and a relatively higher number of neurons within slices than on their surface. These results support the notion of several studies that environmental cues strongly influence the potential of stem cells (e.g. Fricker et al., 1999). Due to the cut and consequently injured surface of the organotypic culture it was covered by a layer of astrocytes of which after several days and at the time-point of cell application some still showed characteristics of activated GFAP expressing astrocytes (Dr. B. Scheffler, personal communication). Possibly they do not support astrocytic differentiation and proliferation, or might even inhibit it or support neurogenesis and in consequence the number of astrocytes was decreased under these conditions compared to the dissociated culture. Again TNC, expressed by GFAP positive astrocytes, could play a role here since it may have an impact on astrocytic proliferation as mentioned

above and supported by our own findings (Holley et al., 2005). Likewise the environmental cues within the slice could enhance neurogenesis as the dentate gyrus of the hippocampus is a neurogenic region and the majority of integrated cells were located in this region. It has been reported repeatedly that neurogenic areas support the differentiation of region specific cell types (Fricker et al., 1999; Suhonen et al., 1996; Shihabuddin ad al., 2000). E.g. only stem cells transplanted into the neurogenic hippocampal granule cell layer differentiated into cells types characteristic for this layer whereas engraftment into other hippocampal regions resulted in the differentiation of cells with astroglial and oligodendroglial phenotypes (Shihabuddin et al., 2000). In addition specific cell loss that inevitably happened in the slice culture subsequent to cutting many neuronal projections could have enhanced the generation of neurons by suggestively changed environmental cues. Analogous differentiation of neural precursors in the adult mouse into the required formerly degenerated cell type was observed within areas of apoptotic cell death (Snyder et al., 1997).

The here used co-culture system of organotypic slices with neural stem cells allows the examination of multiple developmental processes such as migration, differentiation and neurite growth in a complex physiological environment. The importance of the physiological milieu for cell behaviour became evident by the comparison to pure *in vitro* cultures. Furthermore, this system enables a functional analysis such as the investigation of electrophysiological properties (Benninger et al., 2003). The usage of this culture system could thus further enhance the elucidation of the factors that direct designation and behaviour of applied stem cells for therapeutic use.

4.1.4. TN's influence on neurite outgrowth

TNR and TNC both have been implicated in neurite outgrowth by a number of experiments (see introduction). However, most investigations are confined to *in vitro* manipulations. Results from TN function in neurite growth in a physiological environment are rare. Recently a detailed study found tortuosity of pyramidal dendrites in the TNC deficient mouse (Irintchev et al., 2005) and knock-down of TNR expression in zebrafish led to enhanced branching of the optic tract (Becker et al., 2003). Both studies point to a role of TNs in guiding neuronal processes by contact-inhibition. Constitutive expression of TNs by neural stem cells did not alter the growth of neurites

into the slice tissue suggesting that the importance of TNs in neurite growth is associated with its localization in the ECM. Additionally in vitro studies strongly highlighted cell type specific effects of TNs. As the developed neurons on the slice cultures represent an inhomogeneous population of cell types, a minor portion of them might have been influenced by TN expression, undetectable by analyzing the total number. However it is also feasible that differences induced by cell endogen expression can be detected with closer investigations looking at distinct properties of axons and dendrites as the mentioned studies only found alterations in specific details of growth.

4.1.5. TNs influence on neural stem cell migration

Due to its presence at sites of active cell migration TNC had been early proposed to play a role in migratory events (Tan et al., 1987; Tucker and McKay, 1991; Bartsch et al., 1992; Bartsch et al., 1995). We have examined the percentages of cells that under the influence of TN deprivation and expression invaded the organotypic slice.

4.1.5.1. TNC deficiency and migration

We could show that a reduced number of TNC deficient cells were found within TNC deficient slices compared to wild-type controls. This finding points to a migration promoting role for TNC. Such has been suggested previously for granule cells, neural crest cells and dopaminergic neurons (Husmann et al., 1992; Halfter et al., 1989; Ohyama et al., 1998; Tucker et al., 2000). In addition TNC may contribute to the invasive properties of tumour cells (Kleihues et al., 1995; Phillips et al., 1998). Several possible roles of TNC during the migration of cells from the surface of a slice into the deeper layers are possible. First the TNC positive environment could have exhibited guiding properties for the cells. Although the path the applied cells took into the slice did not represent a physiological migration route it is conceivable that the stem cells moved along e.g. TNC positive glial fibers that extended into the slice tissue. However we did not observe differences in the number of invading cells if only the environment was TNC deficient but not the cells themselves. Our finding that the additional deprivation of TNC in the stem cells was necessary to negatively affect migration suggests that the cell endogenous expressed TNC plays an additional or pivotal role in

the investigated behaviour. In comparison it was e.g. the down-regulation of TNC expression in neural crest cells themselves that inhibited their migration (Tucker et al., 2001). The more cell inherent properties of TNC that were of importance here may be related to its described anti-adhesive effect (Bartsch, 1996) which could have initiated easier detachment of the cells promoting the invasion into slice tissue.

Moreover TNC may promote cell locomotion. Focal adhesions between substrate and cell play an important role in supplying the contact between extracellular matrix and actin-based scaffolds in the cell and their turnover is critical for cell motility (Ilic et al., 1995; Ren et al., 2000). TNC has been implicated in these processes. It has e.g. been found to induce loss of focal adhesions in endothelial cells (Murphy-Ullrich et al., 1991). On the other hand TNC has been reported to promote clustering of filamentous-actin, EGFRs, and tyrosine kinases within focal adhesions dependent of the αvβ3 integrin receptor (Jones et al., 1997) thus TNC may support cross-talk between integrins and receptor tyrosine kinases on actin-based scaffolds within focal adhesions (Jones an Jones, 2000). The integrin αvβ3 has not been described in neural stem cells but in oligoprecursor cells (Milner et al., 1997). Possibly it becomes expressed during early stages of maturation of cells and thereby mediates TNC‘s modulatory role in migration. Other integrins may play important roles, such as β1 integrins that have been identified to regulate chain migration of neural precursor cells (Jacques et al., 1998) and have been identified as TNC receptors (see introduction). Thus the loss of direct interaction between integrins and TNC may contribute to the reduced migration of TNC deficient neural stem cells we observed. Receptors in addition to integrins may influence TNC dependent neural stem cell migration. The TNC binding proteoglycan phosphacan e.g. has been localized to radial glial fibers and to migrating neurons suggesting an involvement in neuronal migration (Bandtlow and Zimmermann, 2000). Other TNC binding glycosaminoglycans such as dermatan sulfate and heparin were found to stimulate glioma migration (Aguiar et al., 2005). AnnexinII mediated TNC dependent enhancement of cell migration in a cell culture wound assay and Caric et al. reported that the EGFR mediates chemotactic migration in the telencephalon (2001) suggesting an involvement in the observed effects. TNC has also been found to up-regulate MMPs e.g. in smooth muscle cells (Wallner et al., 2004) which could alter the ECM by controlled proteolysis into a migration promoting environment. In fact involvement of

MMPs in cell motility has been described for several cell types e.g. smooth muscle cells, fibroblasts, cardiac neural crest cells and various tumour cells (Seiki 2002). Important binding partners of MMPs are integrins, which describes a complex pattern of interaction that may play a role in TNC promoted migration.

4.1.5.2. TN expression and migration

Stem cells have great potential in therapy of CNS degenerative diseases or injury. It would be desirable to be able to control and enhance the migration of neural stem cells or precursor cells that are implanted into the adult brain. The finding of a decreased migration of TNC deficient stem cells suggested that TNs could play a role in promoting cell motility which we fathomed by constitutive TN expression. In contrary to our aspiration, constitutively expressed TNs did not alter the motility of cells. Neither the expression of TNR nor TNC led to a changed number of cells within the deeper layers of organotypic slices. TNR was previously found to play a role in migration of olfactory bulb precursors. Its expression at ectopic sites distracted the cells from their normal route. The physiological relevance was suggested to be specifically the initiation of radial migration at the final stage of migration along the rostral migratory stream (Saghatelyan et al., 2004). Probably TNR does not alter cell intrinsic migration properties of the cells but rather plays a role at specific sites of expression.

The absent migration promotive effect of constitutive TNC expression may have several reasons. With the extended survival time of rat slices we investigated an extended period of neural stem cell behaviour. It is possible that the effect observed in TNC deficient cultures only mirrored a slowed migration and the resulted difference in the number of invading cells had been adjusted by the investigated time-point, four days later, in rat slices. In addition differences in mouse and rat slice tissue may underlie the lack of promoted migration. Conceivable is also that the cells in slice cultures migrated at their highest rate and despite a regulatory role of TNC in cell migration a further promotion is not possible due to other limiting factors.

TNC exhibited effects on cell migration as its deficiency has led to decreased numbers of cells that invaded the slice tissue. Our anticipation to further promote cell locomotion by constitutive TN expression was not fulfilled. Yet we tested the effect of TNC expression in the TNC positive hippocampus. It will be interesting to find out whether

TNC expression will promote migration in a TNC negative environment which could later on contribute to an improved distribution of therapeutically used stem cells implanted into adult TNC negative brain regions.

4.1.6. What do the experiments tell us about the function of TN during differentiation?

4.1.6.1. TNC deficiency and differentiation

Using the co-culture system of TNC deficient mouse slices and neural stem cells and their wild-type littermates we could not support our result from the dissociated cultures that showed an increase in the number of astrocytes in the absence of TNC. The slice culture represents a far more complex situation resembling the *in vivo* conditions with a number of binding partners of TNC present e.g. proteoglycans or integrins that could block binding sites that in dissociated cultures have been available. In addition possible changes did not become apparent due to parallel altered proliferation, maturation or cell death that balanced each other. Regulation of other proteins within the slice may have been compensated and overcame the TNC deficiency that in dissociated cultures led to alterations. At this point we have to come back to our suggestion that the lower percentages of astrocytes on organotypic cultures may be due to TNC function in controlling proliferation. Because we did not observe differences in the number of astrocytes between TNC-/- and TNC+/+ slices we have to argue now that if the reactive astrocytic top layer has an impact on astrocytic differentiation of the applied cells it will be at least mainly mediated by other cues than TNC. TNC may exert its effects by modulating other extracellular factors as it was suggested in respect to PDGF induced proliferation of oligodendrocyte precursors (Garcion et al. 2001) or BMP-4 (Garcion et al., 2004). BMP-4 stimulation of CNS precursors *in vitro* enhanced gliogenesis (Gross et al., 1996). If e.g. in TNC deficient dissociated cultures astrogliogensis was enhanced due to the missing negative regulation of BMP-4 signalling by TNC, this signalling pathway may have not played a role in slice cultures e.g. due to a lack of the mitogen or other interacting molecules blocked the crucial binding site of TNC leading to inefficiency of TNC.

4.1.6.2. TN expression and differentiation

The constitutive expression of TNs in neural stem cells has painted a new picture. Ectopic TNR expression did not alter the numbers of oligodendrocytes, astrocytes or neurons. This result now strengthens the *in vitro* finding of TNR deficient stem cells and suggests that TNR expression does not influence neural stem cell differentiation. As mentioned above differences in rapidity of maturation may have been failed to be noticed due to a later analyzed time-point.

In contrary TNC over-expression respectively ectopic expression in subsets of the culture decreased the number of mature neurons. This could have been the result of altered maturation, probability of cell fate, proliferation or cell death. The finding that the number of β-tubulin positive cells including early commited neurons was not altered but the number of NeuN positive mature neurons was decreased, speaks for a role of TNC in the maturation or survival of neurons. This again contradicts with the results of Garcion et al. (2004). Although their described enhanced neurogenesis of TNC-/- cells could match our finding under TNC over-expressing conditions unlike us they see differences by using β-tubulin as a marker. As stated above methodological differences may account also for these contradictions. TNC is expressed by subsets of neurons such as motoneurons and interneurons. It will be interesting to investigate whether the difference in neuronal cell number can be attributed to changes in specific neuronal subclasses. Could differences in migratory potential account for the result? We can preclude this possibility because we were able to replicate the results from slice culture in dissociated cultures. Thus two possible explanations remain: First TNC expression could inhibit the maturation of neurons or reduce their survival. The analysis of β-tubulin positive cells showed a slight increase (approximately 2 %) in case of constitutive TNC expression. Though this difference did not reach significance it could account for the reduced number of NeuN positive cells speaking for a halt of neuronal maturation. As mentioned repeatedly, TNC has been described to modulate growth factor signalling, e.g. to enhance sensitivity to bFGF and PDGF and inhibit BMP-4 signalling (Chung et al., 1996; Jones and Rabinovitch, 1996; Garcion et al., 2004). BMP-4 stimulation of CNS precursors *in vitro* was found not only to enhance

gliogenesis, as mentioned above, but additionally to enhance maturation of neurons (Gross et al., 1996; Mabie et al., 1999). What leads to these diverse effects of BMP-4 signalling is currently not clear, but cell inherent properties and availability of interactions assumingly influence its function. Accordingly, if e.g. TNC regulated astrogliosis by influencing BMP-4 signalling under conditions present in dissociated cultures (as we suggested above), it is conceivable that a changed availability of TNC binding sites or interaction partners in slice cultures in conjunction with TNC over-expression now modulated the maturation of neurons. However, the role TNC has been found to play in growth factor signalling could also lead to a different scenario with final apoptosis: Possibly the high and ectopic TNC expression in neurons displayed a mitogenic signal which conflicted with the differentiation signal. It is established that conflicting growth and differentiation signals initiate apoptosis, which has its importance in tumour suppression when cell suicide is initiated in response to deregulation of growth control. For instance expression of the growth promoting E1A adenoviral gene in serum-free conditions induced apoptosis (Sawada et al., 1995). As mentioned above TNC could lead to an up-regulation of MMPs. This up-regulation led to cleavage of TNC resulting in an accumulation of EGFL domains which in turn were found to induce apoptosis in these cells (Wallner et al., 2004). Several MMPs e.g. MMP9 are expressed in neurons (Backstrom et al., 1996). Possibly they function in a similar way in our culture system supporting the idea that enhanced apoptosis underlies the reduced neuronal numbers.

Why did we not observe a changed number of NeuN positive cells on TNC deficient slices? As mentioned in connection to migration differences in mouse and rat tissue or differences in the analyzed time-point could underlie this controversion. Alternatively TNC functions may vary depending on the level of its concentration. TNC is endogenously expressed by neural stem cells and its expression to excess may have induced mechanisms that were inactive at lower concentration levels in the wild-type situation. This view is further supported by the finding that to the contrary of TNC deficient cultures TNC constitutive expression did not alter the number of astrocyte in dissociated cultures.

Focusing on differentiation processes our results suggested that TNC plays a role in regulating the number of astrocytes and neurons. The conflicting results in dissociated

and slice cultures, deficient and constitutive TNC expressing cells may be explained by differences in the experimental conditions. In conclusion, TNC exerts diverse cell specific functions that moreover depend on additional interactions, factors or on its concentration levels

4.1.7. TNC influences EGFR expression

TNC function could be mediated by extracellular interaction of TNC with mitogens or other factors that potentiates or inhibits their effects or by binding to cell surface receptors subsequently affecting signalling pathways. Several receptors for TNC that might be involved in regulation of cell growth, maturation or migration have been described, among them annexinII which has been reported to mediate a mitogenic effect on endothelial cells and to enhance cell migration (Chung et al., 1994, 1996), the αvβ3 integrin that was shown to modulate EGF-driven growth responses of smooth muscle cells and to mediate effects on proliferation in oligodendrocyte progenitors (Garcion et al., 2001), cell surface phosphatases such as members of the receptor-like protein tyrosine phosphates family (Milev et al., 1997), or the EGFR that mediates growth factor responses and migration in certain brain regions (Caric et al., 2001; Prenzel et al., 2001; Swindle et al., 2001). Caric et al. e.g. found the EGFR to mediate chemotactic migration in the telencephalon (2001). We started the investigation of involved players by analyzing the expression pattern of the EGFR in correlation to TNC expression during the differentiation of neural stem cells. The expression level of the EGFR prior and after the induction of differentiation in dissociated cell cultures was determined and revealed that TNC expressing cells show higher levels of EGFR protein in comparison to the control cells after four days of differentiation.

4.1.8. Could the EGFR regulation account for TNC effects on neural stem cells?

At early developmental stages (mouse E11) TNC was shown to promote EGFR expression by stem cells of the subventricular zone (Garcion et al., 2004). The neural stem cell population we obtained from E14 animals represents a heterogeneous culture of EGF and bFGF responsive cells (Tropepe et al., 1999). Furthermore EGFR

expression was detected in astrocytes, oligodendrocytes and neurons including neurons from dorsal root ganglia, cerebellum and cortex (Gomez-Pinilla et al., 1988; Huerta et al., 1996). At this point we cannot discriminate whether all or only subsets of cells were induced by TNC to up-regulate or extend the EGFR expression. However, it is possible that β-tubulin positive cells were influenced to up-regulate EGFR expression and EGFR signalling may exerted effects on the stage of differentiation e.g. it has been reported to stabilize β-tubulin mRNA (Jinno et al., 1988). Keeping with this hypothesis TNC may prevent premature neuronal differentiation by EGFR regulation. As the EGFR expression was detected in astrocytes of young mice (Gomez-Pinilla et al., 1988), EGFR regulation could also have played a role in increasing the number of astrocytes in TNC deficient cultures.
As described above the EGFR may play a role in migration processes thus presuming that the up-regulated or prolonged expression in culture was brought about by migratory progenitor cells the regulation of the EGFR doubtless could be involved in the observed reduced invasion of TNC over-expressing stem cells into slice cultures. Further analysis e.g. by applicating EGFR deficient cells will clarify this issue.

What could lead to the up-regulation of the EGFR? Garcion et al. (2004) suggested an enhancement of bFGF respectively inhibition of BMP-4 signalling by TNC modulates expression of the EGFR. Although the culture medium was deprived of growth factors a basic level of these factors may have been produced by radial glia cells and progenitor cells within the culture, which were described to produce BMP-4 and bFGF, respectively (Lillien and Raphael, 2000). Thus, once again an implication of factors such as BMP-4 is likely.

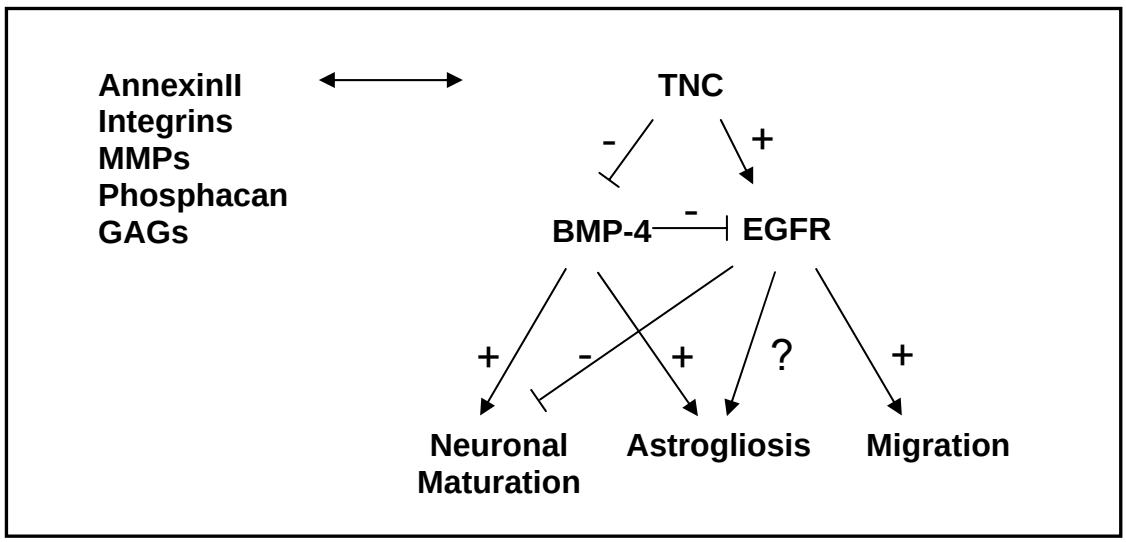


Fig. 26 Proposed TNC functions and interactions
The scheme summarizes our findings and possible involved interactions. We are assuming here that it is neuronal maturation that led to a reduction of NeuN positive neurons and astrogliosis that led to higher numbers of astrocytes. In all three processes TNC could function by positively regulating the expression of the EGFR either directly or by negatively influencing BMP-4 that in turn inhbits EGFR expression. BMP-4 may also by itself interfere with neuronal maturation or astrogliosis. Several more interaction partners of TNC may play a role, some of them depicted in the scheme. Note that signalling induced by additional peptide factors has been suggested to be modulated by TNC which are omitted in this scheme.

4.1.9. Conclusion

Our data suggest that TNC plays a role in the regulation of astrocytic numbers, maturation or apoptosis of neurons and migration of neural stem cells or their descendants. We also find that the experimental conditions exhibit a great influence on TNC functions pointing to a role for TNC in influencing growth factors signalling or modulating functions of other surface and matrix bound molecules. TNR did not exhibit any effects in our experiments which does not necessarily mean that it has no effects but our experimental set-up was not suitable to detect such.

The effects TNC exerted on the development and behaviour of neural stem cells could be mediated by positively regulating EGFR expression, as we could observe increased EGFR levels in constitutively TNC expressing cells (Fig. 26). Concluding we like to

hypothesize on the basis of our results that TNC expression is important for the migration of progenitor cells and its expression along the pathways of migrating neuroblasts may inhibit their premature differentiation into mature neurons (Fig. 27).
It will be enlightening to perform the investigations, such as those suggested at corresponding sites in the text, which will be necessary to validate the proposed functions.

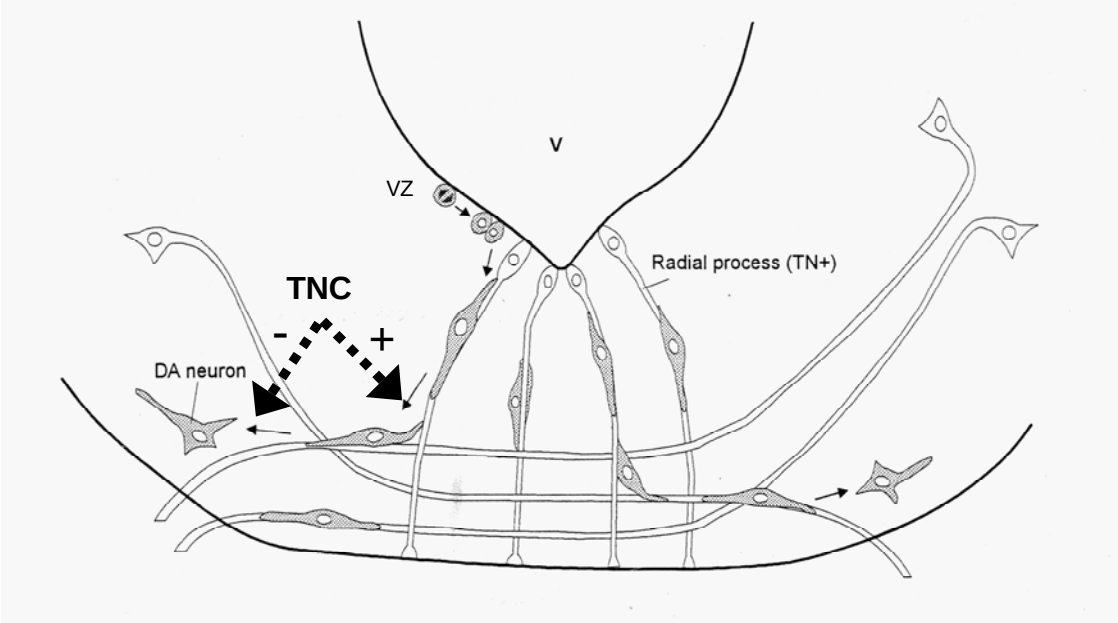


Fig. 27 Hypothesized function of TNC
Schematic drawing of the migration of dopaminergic (DA) neurons in mice.
DA neurons are generated in the VZ, migrate along TN-immunoreactive processes of neuroepithelial cells and laterally along tangentially arranged nerve fibers (From Ohyama et al., 1998).
Our results support the here suggested role of TNC (scattered arrows) in enhancing or guiding (+) migration of early committed neurons and additionally point to a role in preventing (-) untimely neuronal maturation. V: ventricle

4.2. Transgenic mouse

We had attempted to yield a transgenic mouse line over-expressing TNC in astrocytes by usage of the human GFAP promoter. Although three founder lines showed higher levels of TNC mRNA no protein was detectable by Western blotting or immunohistochemistry after crossing into the TNC negative background. The detection of higher mRNA levels in the transgenic animals pointed to a functional promotor. As we aimed to produce an over-expression of protein possible feedback mechanisms could lead to a quick equalization of protein levels but since we could not detect any protein in the TNC deficient background this explanation is ruled out. Possible is that for some unknown reason not the entire transgene inserted into the genome. The produced aberrant transcript was recognized by the primers used in real-time PCR but was not sufficient to induce a correct translation because of the lack of the signal for the start of translation or because of an introduced frame shift. Due to the numerous splice variants of TNC it will be difficult to detect the transgenic transcript in northern blot analysis. To find the hindrance it would be necessary to sequence PCR products of the inserted transgene. In a second attempt to construct TNC over-expressing mice it will be advantageous to use a tag that will allow easy detection of transcript and protein.

Study two: Pathfinding errors of the corticospinal tract

1.0. Introduction

A crucial step in the correct wiring of the nervous system is the guidance of nerve fibers towards their appropriate synaptic targets. The precision is achieved by two types of early acting mechanisms independent of neural activity and later-acting, activity-based refinement mechanisms (Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996). For the former mechanism the hand-like structure on the tip of growing neurites, the growth cone, is essential, it bears receptors for and interacts with many signals it encounters on the way towards its target. The growth cone is composed of lamellipodia, which contain cross-linked networks of actin-filaments, and filopodia, tensile structures, composed of a bundle of filamentous actin that probe the extracellular environment. This peripheral actin network is associated in the proximal portion of the growth cone with microtubules located in the axon shaft and contributes to the assembly and translocation of microtubules. Thus, the regulation of actin polymerization at the

leading edge of filopodia and lamellipodia and actin depolymerization in proximal regions of the growth cone is essential for the control of growth cone advance or retraction (Huber et al., 2003).

1.1. Growth cone guidance

A classical view groups mechanisms that guide growth cones to their targets into chemorepulsion, chemoattraction, contact-dependent repulsion and contact-dependent attraction. However, elucidating the mechanisms reveals that guiding molecules do not necessarily follow such a scheme. A diffusible long-range chemotropic factor might be transformed into a locally acting, contact-dependent factor by binding to components of the extracellular matrix or to the cell surface (Serafini et al 1994; Maclennan et al., 1997; Deiner et al., 1997). Non-diffusible molecules may be expressed in a spatially graded manner across a zone and may then act as chemotropic guidance cues in a manner similar to diffusible chemotropic factors. For neurotrophins, neurotransmitters and netrins it was shown that a single growth cone can respond to the same diffusible cue with an attractive or a repulsive response, depending on the level of cyclic AMP (cAMP) in the growth cone (Ming et al., 1997; Song et al., 1997, 1998). Whether a growth cone is repelled or attracted is therefore due to intrinsic features of a guidance cue but is additionally determined by the state of the responding growth cone, e.g. the level of second messengers.

1.2. Guidance molecules and guidance receptors

The intracellular regulators of cytoskeletal rearrangements involved in axon guidance cue signalling are members of the Rho family of small GTPases such as Rac, RhoA or Cdc42. In addition to neurotrophins, neurotransmitters and netrins, other extracellular cues implicated in guiding neurites are ephrins and semaphorins. Another prominent group of molecules that serve as guidance cues and receptors in axon growth, axon fasciculation, and axon guidance are cell recognition molecules. There exist three main families of molecules, the integrins, the cadherins and the Ig superfamily. Characteristic for members of the Ig superfamily is the existence of at least one Ig-like domain, which

allows them to mediate cell adhesion via a calcium independent mechanism (Brummendorf and Rathjen, 1993).

1.2.1. The L1 family of cell recognition molecules

The L1 family is a small subfamily within the Ig superfamily of cell recognition molecules. The founding member is L1 in mouse and human and species homologues include L1.1 and L1.2 in zebrafish (Tongiorgi et al., 1995), neuroglian in invertebrates (Bieber et al., 1989), neuron-glial cell adhesion molecule (NgCAM) in chicken (Grumet et al., 1984) and NILE in rats (Salton et al., 1983). Additionally, three closely related

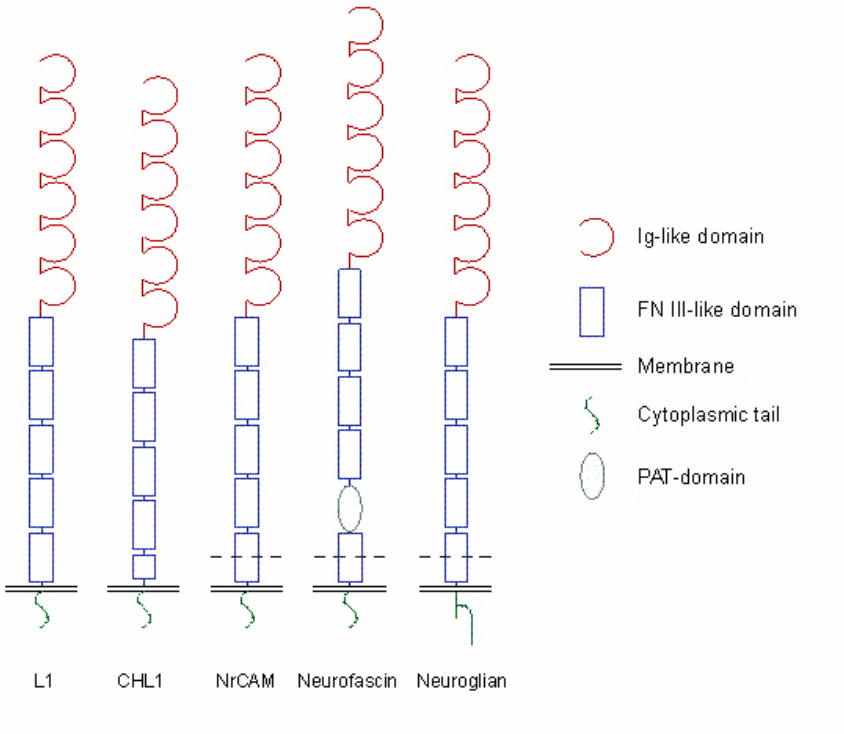


Table 1 Modular structures of the different members of the L1 family
The distinct modular structures of the L1 family members are displayed in this schematic drawing. The domains are specified in the list next to the scheme. PAT stands for proline-, alanine- and threonine rich domain.

proteins have been identified, which are the close homologue of L1 (CHL1) (Holm et al., 1996), NgCAM related cell adhesion molecule (NrCAM) (Grumet et al., 1991; Kayyem et al., 1992), and neurofascin (Volkmer et al., 1992).

All members of this family share a similar modular structure of six Ig-like domains, at least four FNIII-like domains, a transmembrane stretch and a highly conserved cytoplasmic tail (Table 1). A binding site for ankyrin within the intracellular portion connects L1 with the spectrin cytoskeleton.

In the developing central nervous system L1 is highly expressed on axons of postmitotic neurons and on their growth cones in all brain regions, including the pyramidal tract (Joosten and Gribnau, 1989; Cohen et al., 1998; Dahme et al., 1997; Demyanenko et al., 1999; Fujimori et al., 2000). Homophilic and heterophilic interactions of L1 (in cis and trans) can regulate neuritic growth (Brummendorf and Rathjen, 1996) by activating several signalling pathways through second messenger cascades (Burden-Gulley et al., 1997), also driving cytoskeletal rearrangements (Davis and Bennett, 1994; Burden-Gulley et al., 1996). A crucial role of L1 for normal brain development is highlighted by the fact that mutations in the human L1 gene, located on the X chromosome in Xq28, cause a severe neurological disease, termed CRASH (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus; (Fransen et al., 1995). The clinical picture of CRASH patients is complex and variable. Hypoplasia of the corticospinal tract (CST) or pyramidal tract and corpus callosum, adducted thumbs, mental retardation and hydrocephalus are among the symptoms of the disease (Brummendorf et al., 1998; Fransen et al., 1995; Kamiguchi et al., 1998). An analysis of knock-out mice lacking the L1 molecule has confirmed an essential role for L1 in CST development. Most axons remain ipsilateral at the pyramidal decussation and do not extend to the contralateral side as in wild-type animals (Fig. 1) (Cohen et al., 1998; Dahme et al 1997; Demyanenko et al., 1999; Fransen et al., 1998).

In case L1 is necessary for the correct guidance of one of the major cortical projections, the CST, which environmental cues or receptors is L1 interacting with? Two such possible candidates are the Semaphorin3A (Sema3A; Raper and Kapfhammer, 1990; Luo et al., 1993) and CD24 (Springer et al., 1978; Fischer et al., 1990), which have been implicated in neurite guidance and will be introduced below.

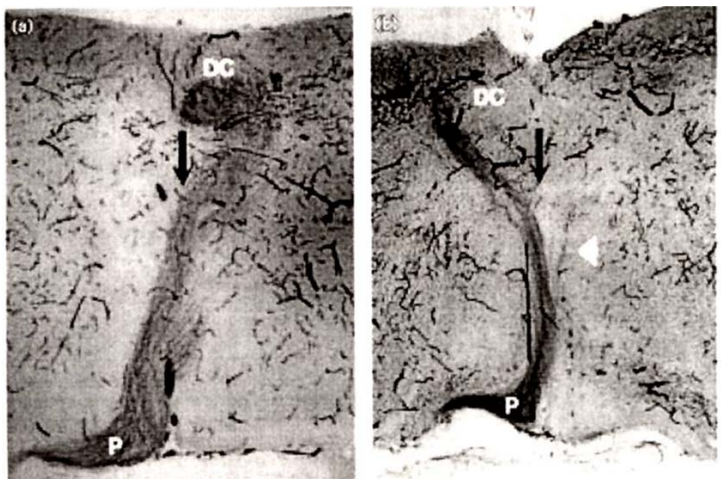


Fig. 1 Pathfinding errors of the CST in L1 deficient mice
Coronal sections of the pyramidal decussation. The left picture shows the wild-type pyramidal tract (P) that runs from ventral (bottom) left to dorsal (up) right (forming the dorsal column, DC) thereby crossing the midline (arrow). On the right is shown that under L1 deficient conditions the size of the tract is decreased. Only a few fibers (arrowhead) cross the midline. The majority of axons stays ipsilateral.
From Cohen et al., 1998

1.2.2. Semaphorins

Sema3A is a member of a large group, the semaphorins, which all are implicated in developmental events. The group consists of at least 30 different members that share a domain of about 500 amino acids (Inagaki et al., 1995; Chen et al., 1998). This homologous domain, called sema-domain, is found in proteins from vertebrates, invertebrates and even viruses. Based on sequence comparisons of this domain and structural similarities, semaphorins were grouped into currently seven classes (Semaphorin Nomenclature Committee, 1999).

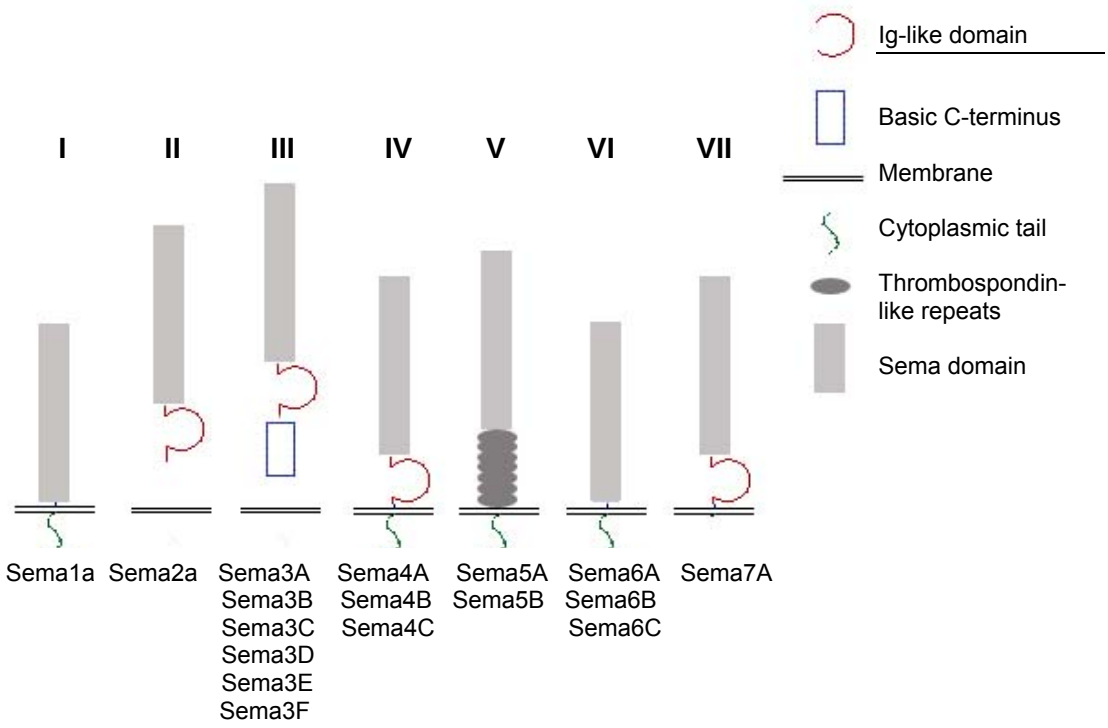


Table. 2 The Semaphorins are devided into seven classes, of which some members are listed. The domains are specified in the list next to the scheme.

Classes I and II contain only invertebrate members, whereas classes III-VII contain vertebrate. The extracellular 480-500 amino acid sema-domain in semaphorins is preceded by the N terminal signal peptide and followed by a class specific C-terminal domain of 70-500 amino acids. Secreted proteins have a highly charged carboxy terminus and are grouped into classes II and III whereas all other classes contain transmembrane proteins. A C2 type Ig-like domain located carboxyterminally to the sema-domain is found in semaphorins of classes II-IV (Mueller, 1999) (Table 2).

The first observation of semaphorin action was a contact mediated retraction of growth cones. Sympathetic growth cones crossing retinal neurites resulted in a retraction of the growth cone (Kapfhammer and Raper, 1987). A protein that was extremely potent in inducing growth cone collapse of chick dorsal root ganglia was purified from membrane fractions of the chick brain and cloned. Due to its repulsive action it was named collapsin (now renamed Sema3A; Luo et al., 1993).
In combination with the grasshopper fasciclin IV/SemaI/now Sema4C (Kolodkin, 1992; Luo et al., 1993) Sema3A is the founding member of the semaphorins. Semaphorins were found to play a role in nervous system development. E.g. Sema4C acts to cause

fasciculation and has also been shown to bundle non-fasciculating axons when expressed ectopically (Tessier-Lavigne and Goodman, 1996). Sema2a controls synaptogenesis and growth cone arborization (Matthes et al., 1995). Sema1b works to steer growth cones (Fan and Raper, 1995), Sema4D inhibits axonal growth and is expressed by oligodendrocytes and up-regulated after CNS lesion, suggesting a role for semaphorins in regeneration (Moreau-Fauvarque et al., 2003) or oligodendrocyte guidance (Cohen et al., 2003). Additionally, semaphorin genes code for proteins that have been implicated in development of bone (Togari et al., 2000) and vasculature (Shima and Mailhos, 2000), cancer metastasis (Xiang et al., 1996), B-cell aggregation and differentiation (Hall et al., 1996).
Class III semaphorins are the most extensively studied. In conjunction with Sema3A they are not only the prototype of the semaphorin family, but are also the only secreted vertebrate semaphorins.

1.2.3. Semaphorin3A

Sema3A is a secreted protein of 100kD. Thus, it lacks a transmembrane domain although it has a highly basic region near its C-terminal end. It is extensively glycosylated with an Ig-like domain following the sema domain (Luo et al., 1993; Kikuchi 1997).
The repellent response induced by Sema3A action is accompanied by a dramatic reorganization of the cytoskeleton (Fan et al., 1993) and can also be elicited by purified or recombinant Sema3 (Luo et al. 1993). The collapse of the growth cone might be an unphysiological effect; when bound to localized substrate Sema3A can steer the extension of sensory axons *in vitro* by causing a deflection of the original pathway without full collapse of the growth cone (Fan et al., 1993; Fan and Raper, 1995).

1.2.3.1. Sema3A expression and functional implications

Sema3A transcript is expressed in the embryo from E9.5. At early stages it is e.g. expressed by mesodermal cells, later by different classes of neurons such as cerebellar Purkinje cell and motoneurons (Catalano et al. 1998). During the development Sema3A expression is regionally restricted and often appears stripe-wise, which suggests that it

plays a role in shaping cell regions and channelling axons and nerves. It may e.g. guide sensory and motor axons through the anterior part of the somite (Keynes and Stern, 1984, 1988; Adams et al., 1996) and separate individual dorsal root ganglia (Püschel et al., 1995; Adams et al., 1996). Sema3A mRNA is found in the ventricular zone of the developing cerebral cortex, as well as in the cortical plate and the striatum. In the cerebellum Sema3a mRNA is detected in mouse at E16 in alternating stripes of Purkinje cells until P16, when all Purkinje cells express Sema3a (Catalano et al., 1998). Consistent with the cerebellar expression of Sema3A *in vitro* studies show an inhibition of growth cones of basilar pontine axons, axons that develop as mossy fibers *in vivo* (Rabacchi et al., 1999) indicating that Sema3A prevents mossy fibers to innervate inappropriately Purkinje cells. Semaphorins are expressed differentially in the hippocampus and Sema3A and Sema3F were shown *in vitro* to repel hippocampal axons (Mark et al., 1997; Chedotal et al., 1998).

Sema3A is also expressed in a subset of deep nuclei neurons and all cranial motor neurons (Catalano et al., 1998). At the same time cranial motor axons such as trochlear, trigeminal, facial, glossopharyngeal axons as well as spinal and abducens motor axons were repelled by Sema3A secreting cell clusters (Varela-Echavarria, et al., 1997).

In the spinal cord Sema3A is first expressed throughout the spinal grey matter, and later disappears from the dorsal region whereas expression persists in the ventral region which supported a role for Sema3A in the proper entry of collateral sensory branches with different Sema3A sensitivities into the spinal cord and their termination in different laminae (Messersmith et al., 1995; Fu et al., 2000). Most importantly, Sema3A expressed by ventral spinal cord explants repels cortical axons pointing to a role of Sema3A in motor axon guidance (Castellani et al., 2000). Interestingly it additionally acts as a chemoattractant on their apical dendrites. For chemoattractive effects, an asymmetrically localization of guanylate cyclase was required (Polleux et al., 2000). These observations widened the view of the action mechanism of Sema3A from the more one-dimensional picture of a typically repellent factor to a complex mode of functioning involving different receptors, coreceptors and the biochemical state of the cell.

1.2.3.2. *In vivo* findings

To investigate the *in vivo* effects of Sema3A two mutant mouse lines, deficient for the Sema3A gene, have been created in two independent laboratories (Behar et al., 1996; Taniguchi et al., 1997). Their analysis could partly underscore *in vitro* findings, though the observed effects were often milder than expected and studies of both mutant lines frequently resulted in differing outcomes.
An impact of Sema3A deficiency on the patterning of sensory projections in the spinal cord could e.g. be supported by a deletion study in which some sensory afferents in the homozygous mutant mice projected outside their normal termination field (Behar et al., 1996). In contrast, this effect could not be observed in the second mutant mouse, which exhibited normal projections of sensory afferents (Taniguchi et al., 1997). Furthermore, Behar et al. found misorientated processes in the cerebral cortex and a reduced thickness of the cortex in homozygous mutant animals. However, many major projections, including climbing fiber, mossy fiber, thalamoocortical, basal forebrain projections and cranial nerves developed normally in these Sema3A deficient mice (Catalano et al., 1998). The most severe defects were found in bone and cartilage development (Behar et al., 1996). On the other hand, Taniguchi et al. (1997) could not observe any defects in the central nervous system, but peripheral nerve projections showed severe abnormalities. The cranial nerves in homozygous embryos tended to defasciculate and spread over a larger area in the termination field. Occasionally individual axons projected aberrantly as was observed for the peripheral projections of sensory neurons. However, as mentioned above the trajectory of sensory afferents in the spinal cord was normal. In the absence of Sema3A the shape of the sympathetic chains was disrupted with cell bodies scattered over a broader area (Taniguchi et al., 1997).

Although dissimilar, these results suggest that Sema3A is an important player in the guidance of certain axons. The removal of the repulsive cue enabled axons to grow aberrantly into normally not invaded regions; a normally small growth corridor widened leading to spreading of axons, and resulting in defasciculation because axon-axon interactions were no longer favoured over axon-substrate interactions.

1.2.3.3. Sema3A interactions

Two groups independently identified a transmembrane protein called neuropilin-1 as a Sema3A receptor or a component of a receptor complex (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin-1 is a cell-surface glycoprotein with three unique, highly conserved extracellular domains. Its expression is restricted to axons of particular neuron classes and coincides with times of axonal growth (Kawakami et al., 1996). Interestingly, neuropilin-1 deficient mice show a similar phenotype as described by Taniguchi et al. (1997) concerning the Sema3A mutant mouse (Kitsukawa et al., 1997; Kawasaki et al., 2002). Moreover, dorsal root ganglia growth cones from the neuropilin deficient mouse are insensitive for Sema3A, proving the necessity of the neuropilin-1 receptor for collapse induction (Kitsukawa et al., 1997).
Neuropilins and semaphorins are believed to form receptor complexes that involve other receptors, the plexins. Plexins are related to the vertebrate semaphorin family. They all contain the sema domain. So far no plexin has been found to directly bind to Sema3A but plexin-A1 complexes with neuropilin-1 as a coreceptor. The neuropilin-1 / plexin-A1 complex was found to have a higher binding affinity to Sema3A than neuropilin-1 alone and seems to be the physiologic Sema3A receptor (Takahashi et al., 1999). The cytoplasmic domain of plexins associate with tyrosine kinase activity. Deletion of this highly conserved domain of plexin-A1 or –A2 renders former Sema3A sensitive sensory or spinal neurons resistant to its repulsive effects suggesting that functional semaphorin receptors contain neuropilins as ligand-binding units and plexins as signal-transducers (Tamagnone et al., 1999; Rohm et al., 2000).

Another component of this complex may be L1. As mentioned above Sema3A released by the ventral spinal cord repels cortical axons (Castellani et al., 2000). The finding that L1 deficient neurons failed to respond to Sema3A and that L1 and neuropilin-1 were coimmunprecipitated indicated a cross-talk between Sema3A and L1 that may be important for axonal pathfinding (Castellani et al., 2000) (Fig. 2).
Downstream signal transduction components that transmit semaphorin cues into intracellular events include a family of proteins (collapsin-mediated response proteins,

CRMP's) of which CRMP2/TOAD64 plays a critical role during development (Wang and Strittmatter, 1996).

1.2.4. CD24

CD24, nectadrin or heat stable antigen (which it was named because of its resistance to denaturating heat after fixation, Springer et al., 1978) is a mucine-like glycoprotein, attached to the cell surface via a GPI (glycosyl pohosphaditylinositol)-anchor. Its protein core comprises only 30 aminoacids (about 3 kD), but with extensive O- and N-glycosylations that vary with cell type. The developmental stage CD24 reaches a molecular weight of approximately 27 kD to 79 kD depending on developmental stage and cell type (Fischer et al., 1990; Kadmon et al., 1992, 1995).

1.2.4.1. CD24 expression and functional implications

CD24 was described as a cell surface antigen of immature cells of major haematopoietic lineages. Here it has been used to stage the maturation of B and T lymphocytes because it is strongly induced and then repressed during maturation. *In vitro* studies implicated an involvement of CD24 in cell-adhesion between B-cells but also in activating T-lymphocytes during an immune response. Lack of CD24 in mutant mice could partly underscore these findings; the mice show a leaky block in B-cell development and alterations in erythrocytes, but with no effects on the immune function (Wenger et al., 1995; Nielsen et al., 1997). CD24 expression was additionally detected in epithelia of intestinal mucosa, salivary gland, bronchus, kidney, hair follicle, during tooth development and in the nervous system (Shirasawa, et al., 1993).
Three CD24 forms of 27, 30 and 33 kD are found in the nervous system (Kadmon et al., 1995). Expression was detected in the spinal cord, hindbrain, midbrain and forebrain from embryonic day 11 to postnatal day 10 with a peak between E17 and P5. In the developing spinal cord CD24 is highly expressed at the ventral midline at the pyramidal decussation, where CST axons cross to the contralateral side suggesting an involvement in guidance of the CST (Cohen et al., 1998). In the adult expression was dramatically down-regulated but persisted in the hippocampus, cerebellum and subventricular zone (Nedelec et al., 1992; Shirasawa et al., 1993; Shewan et al., 1996; Belvindrah et al.,

2002). In the cerebellum CD24 is expressed by granule cell progenitors, newly formed parallel fibers and unmyelinated axons (Kuchler et al., 1992). Various other cell types have been shown to express CD24 in the developing mouse nervous system, among them retinal ganglion cells, dorsal root ganglion cells and hippocampal pyramidal cells (Shirasawa et al., 1993). Expression of CD24 by migrating neuroblasts, postmitotic neurons and in developing axonal tracts suggested an implication of the molecule in migration or neurite outgrowth (Shirasawa et al., 1993; Shewan et al., 1996). Neuronal migration in cerebellar cultures was inhibited by CD24 antibodies (Lehmann et al., 1990). *In vitro* experiments could demonstrate neurite outgrowth inhibiting effects of CD24 on retinal and dorsal root ganglion cells and promoting effects on cerebellar neurons. The effects were independent of the CD24 expression of the neurite growing neuron, thus most likely CD24 functions via heterophilic binding (Shewan et al. 1996; Kleene et al., 2001). Additionally, CD24 is re-expressed in the peripheral nervous system following injury suggesting a role in regeneration processes (Shewan et al., 1996). Belvindrah et al. demonstrated additionally expression by proliferating cells in the subventricular zone (Belvindrah et al. 2002).

1.2.4.2. *In vivo* findings

So far, most suggested CD24 functions in the nervous system have relied on *in vitro* studies. CD24 deficiency in mutant mice did not alter the gross morphology of the brain. However, the CD24 deficient brains appear narrower which is most likely due to decreased sizes of putamen and hippocampus (M. Lepore 1995, Diploma thesis 1995). Furthermore, a suggested implication in proliferation and neurogenesis in the subventricular zone could be underscored by investigation of the CD24 deficient mouse (Belvindrah et al., 2002; Nieoullon et al., 2005). The high expression of CD24 in the ventral spinal cord at the region where the CST decussates the midline (Cohen et al., 1998) suggests that the *in vitro* demonstrated L1 mediated repellent actions of CD24 play a role in guiding corticospinal axons.

1.2.4.3. C24 Interactions

CD24 was described to mediate cell adhesion via homophilic and heterophilic binding in *cis* and *trans* position (Kadmon et al., 1992, 1995). Potential interaction partners include the cerebellar soluble lectin (CSL), (Lehmann et al., 1990), the lymphocyte function-associated antigen1 (LFA-1), (Kadmon et al., 1994), the very late antigen (VLA-4), (Hahne et al., 1994) and the cell adhesion molecule L1. CD24 was found to be coexpressed and co-redistributed with L1 (Kadmon et al., 1995). Purified CD24 bound to L1, and antibodies against both lead to an enhanced increase in intracellular Ca^{2+} (Kadmon et al., 1995). A functional importance of this interaction could be shown in cell culture experiments: When using CD24 and L1 deficient neurons, it could be shown that CD24 as a substrate inhibits neurite outgrowth of dorsal root ganglion cells and promotes neurite growth of cerebellar neurons via *trans*-interaction with L1 on the surface of the neurite outgrowing cell. Co-distribution of L1 and CD24 isoforms suggested that only the 30- and 33 kD form of CD24 take part in this binding. Furthermore, this interaction is dependent on certain sugar chains on the CD24 protein core, the sialic acid, which L1 binds to (Kleene et al., 2001) (Fig. 2).

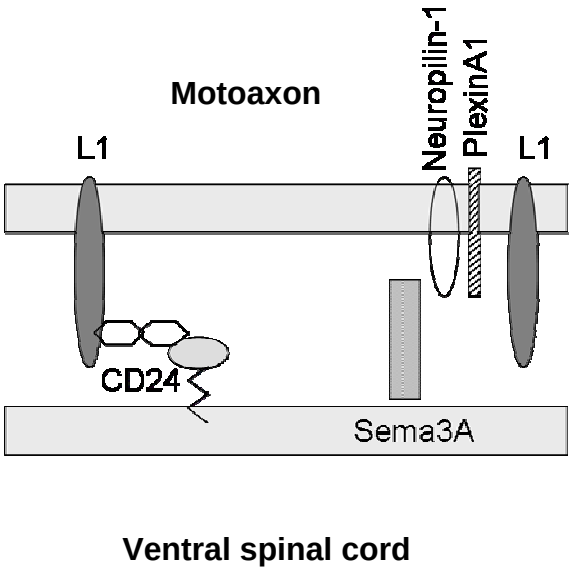


Fig. 2 Suggested interplay between L1 expressed by the growing motoaxons of the CST and CD24 and Sema3A present in the ventral spinal cord. CD24 directly binds to L1 whereas the effects of Sema3A are believed to be mediated by interaction of its receptors neuropilin-1 and plexinA1 with L1.

1.3. The corticospinal tract

The CST (Fig. 3) in the rodent arises from large pyramidal neurons in layer V of the sensory-motor cortex, passes through the ipsilateral medullary pyramid, and subsequently travels in the contralateral dorsal column down to the most caudal levels of the spinal cord. The corticospinal fibers influence the activity of motoneurons in the spinal cord directly or indirectly through the subcortical relay nuclei and spinal interneurons (Terashima et al., 1995).

At the caudal medulla the mass of corticospinal fibers crosses the midline to the contralateral and dorsal side. Only 1 % to 3 % of the neurons in the rat extend axons on the ipsilateral spinal cord (Reinoso and Castro, 1989).

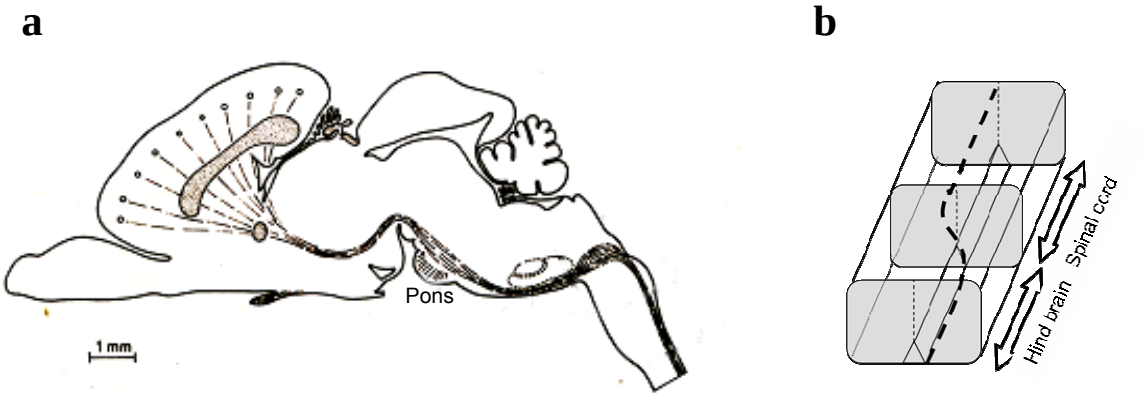


Fig. 3 The Corticospinal / pyramidal tract
a) Drawing of the pathway of the CST in the rodent. The axons arise from pyramidal neurons in the sensory-motor cortex and pass through the internal capsule and pons towards the spinal cord. At several levels collateral branches leave the tract, e.g. in the region of the basilar pons. Before the pyramidal tract reaches the spinal cord the axons cross the midline and coinstantly run to the dorsal side, thereby forming the pyramidal deccussation, which is schematically shown in **(b)**.

Corticospinal neurons project to various subcortical targets such as the superior colliculus, red nucleus, pontine nuclei, inferior olivary complex, and dorsal column nuclei as the tectal, mesencephalic, pontine, inferior olivary and dorsal column-nuclei

collaterals, respectively, providing these subcortical structures with a copy of the motor outflow.

Since the CST in the rodent is an easily identified group of fibers situated in the most ventral portion of the dorsal funiculus of the spinal cord and exhibits considerable postnatal development, it has often been utilized in neurological studies on plasticity and regenerative capacity of the lesioned central nervous system. Because of its prolonged period of postnatal development, the CST represents an advantageous system for studying neurobiology such as path finding, target recognition, collateral formation and elimination and lesion- induced plasticity.

1.4. Aims of study two

L1 is expressed on all axons in the developing nervous system and plays an important role in pathfinding of the pyramidal tract. Its deficiency or ectopic expression leads to severe abnormalities in mouse and human including pathfinding errors of the CST at the pyramidal decussation and in the basilar pons (Cohen et al., 1998; Ourednik et al., 2001).

- Several lines of evidence suggest that Sema3A is a binding partner of a receptor complex comprising L1, neuropilin-1 and possibly plexin1A. Sema3A is expressed in the ventral spinal cord; in co-culture experiments Sema3A has a repulsive effect on wild-type cortical axons but not on L1 lacking axons from mutant animals, implying a role for Sema3A in the pathfinding of cortical descending tracts as the CST (Castellani et al. 2000).

- CD24 has been shown to interact with L1 to increase cell adhesion, raise intracellular calcium and inhibit or promote neurite outgrowth (Kadmon et al., 1995; Kleene et al., 2001). Its high expression at the point of pyramidal decussation suggests an involvement of CD24 in the axonal guidance across the midline (Cohen et al. 1998).

The hypothesized role of CD24 and Sema3A in pathfinding of the CST in particular at the pyramidal decussation and in the basilar pons where L1 deficiency lead to aberrations should be investigated by using *in vitro* and *in vivo* labelling techniques.

2.0. Materials and Methods

2.1. Animals

The generation of Sema3A and CD24 deficient mice has been described (Taniguchi et al., 1997; Wenger et al., 1995). The Sema3A mutant line was kindly provided by Drs. C. Burkhardt and A. Püschel; the CD24 mutant animals were received from Dr. G. Chazal.
Sema3A mutants and wild-type mice were maintained in a C57BL/6J genetic background, and CD24 mutants and wild-type mice in a CD1 background. Both mutant lines were maintained as heterozygous breedings. In order to determine their genotype PCR was routinely applied with the following primer pairs and protocols.

2.2. Genotyping

For methodological details of the general procedure please refer to materials and methods study one.

Sema3A:
The Sema3A PCR was performed as a multiplex PCR according to personal information by Dr. C. Burkhardt. The amplified piece of DNA yields in wild-type animals a size of 1.3 kb and in knock-out animals of 600 bp.

Primer1: P3: 5‘-ATG GTT CTG ATA GGT GAG GCA TGG-3‘
Primer2: P4: 5‘-GTT CTG CTC CCG GCT CTA AAT CTC-3‘

Reaction mix:	PCR protocol:		
1 µl genomic DNA	Cycle 1	94 °	5 min
1 µl 10 pmol primer 1	2	94 °	1 min
1 µl 10 pmol primer 2	3	62 °	30s
1 µl 10mM dNTPs	4	72°	2 min
2 µl 10x-PCR-Puffer	2-4		36 repeats
2 µl $MgCl_2$ (1mM)	5	72°	10 min
0.5 µl Taq			
ad 20µl			

CD24:

Primers and protocol to determine the knock-out animals were adopted from M. Lepore (Diploma thesis 1995). The PCR leads to an amplification of a 1.15 kb sized piece of the knock-out allele. In order to detect heterozygous and wild-type littermates, a PCR that amplifies a 400 bp portion of the wild-type CD24 gene was established.

CD24 wild-type:
Primer 1: CD24_21F: 5'- GCT TAG CAG ATC TCC ACT TAC CGA-3'
Primer 2: CD24_427R: 5'-TTG GAC GGT GGA GGA TGG –3'

CD24 knock-out:
Primer 1: CD24ko1: 5'-CCT AGG TCC GGT TGG ATG-3'
Primer 2: CD24ko2: 5'-AAA GAC TTT GCC CCT CCT-3'

Program wild-type:

cycle	
1	95° 5min
2	95° 45s
3	65° 1min 30s
4	72° 1min
2-4	29 repeats
5	72° 5min

Program knock-out:

cycle	
1	94° 5min
2	94° 40s
3	62° 30s
4	72° 1min 20s
2-4	30 repeats
5	72° 10min

Reaction mix
1 µl genomic DNA
1 µl 10 pmol primer 1
1 µl 10 pmol primer 2
1 µl 10mM dNTPs
2 µl 10x-PCR-Puffer
2 µl $MgCl_2$ (1mM)
0.5 µl Taq
ad 20µl

2.3. Anterograde axonal tracing

Eight days old Sema3A and CD24 mutants and wild-type mice were obtained fixed with 4 % PFA from C.Burkhardt and G. Chazal respectively (the day of birth being defined P0).
A small crystal of the lipophilic fluorescent dye 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, USA) was inserted unilaterally into the medullary pyramid - approximately 1 mm rostral to the pyramidal

decussation. To prevent spreading of the tracer across the midline, the contralateral pyramid was carefully removed with the aid of fine microscissors (Fig. 4a). Brains were stored in 4 % PFA in the dark at 37 °C for at least 4 weeks, subsequently the medulla and cervical spinal cord were serially sectioned with a vibratome.

To anterogradely trace corticospinal axons during development, we used one day old mice from heterozygous breeding pairs. Animals were deeply anaesthetized and the skull was punctured three times with a 27G needle (Braun, Emmenbrücke, Switzerland). DiI was dissolved in dimethylformamide (Sigma, Deisenhofen, D), and about 1 µl of tracer was applied at each injection point with glass micropipettes which were attached to a Multi-Channel Picospritzer (General Valve, Fairfield, NJ) (Fig. 4b). Animals were sacrificed between the second and fifth postnatal day. Brains were fixed by immersion in 4 % PFA and serially sectioned with a vibratome (Leica, Bensheim, D). To analysis the pyramidal decussation, coronal sections were prepared starting at rostral levels of the medulla and ending at caudal levels of the cervical spinal cord. For the detection of pontine collaterals brains were cut sagitally. Analysis was performed on a fluorescent microscope (Axiophot 2, Zeiss, Jena, D).

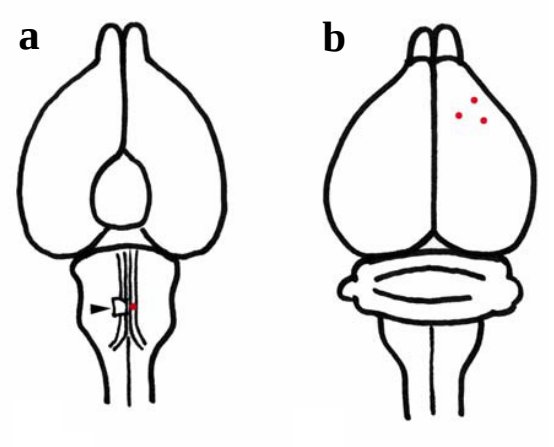


Fig. 4 Schematic drawing of the tracer application site in adult and young animals
The scheme displays a ventral view of an adult brain in **a)** and a dorsal view of a brain in **b)**. In adult animals, the spreading of the tracer is avoided by removing parts of the ventral pyramid contralateral to the application site of the DiI crystal (arrowhead, **a**). The DiI crystal is marked red. In young animals the tracer was injected through the skull at three different points of the motor-cortex (marked red, **b**).

3.0. Results

3.1. Anterograde tracing of the corticospinal tract

The CST originates from pyramidal neurons in layer 5 of the sensory-motor cortex. Corticospinal axons leave the cortex through the internal capsule and pass the basilar pons and the medulla. At the pyramidal decussation axons turn from ventral to dorsal, cross the midline and enter the spinal cord. In the medulla, corticospinal axons form the medullary pyramids at both sides along the ventral midline. At this level, the CST can be identified macroscopically. Macroscopic inspection of the medullary pyramids of P8 Sema3A and CD24 deficient mice and age-matched wild-type animals revealed no significantly reduced size of the tract in the mutants (not shown).

Anterograde tracing of the CST of P8 wild-type and mutant mice (N = 4 for each genotype) with DiI and analysis of the tissue at the pyramidal decussation confirmed no hypoplasia or any aberrations of the tract neither in Sema3A nor in CD24 mutant mice (not shown).

There was no obvious defasciculation of the tract. Corticospinal axons of all mutant mice turned dorsally at the pyramidal decussation, crossed the midline and entered the dorsal column.

3.2. Pathfinding errors of corticospinal axons in young Sema3A and CD24 deficient mice

In mice, the first corticospinal axons reach the pyramidal decussation late on P0 / early P1. The majority of axons arrive during the subsequent days until P3, still followed by late arriving axons (Bastmeyer et al., 1996).

In case the fully developed CST shows no abnormalities, are there any pathfinding errors detectable at earlier stages of development? To investigate this, we performed anterograde tracings of corticospinal axons of early postnatal Sema3A and CD24 mutants and their wild-type littermates.

In a first series of experiments, the tracer was applied at postnatal day one. Eleven CD24 wild-type and 17 CD24 deficient, three Sema3A+/+ and three Sema3A-/- brains were analyzed three or four days later. In the medulla and at the pyramidal decussation, a prominent CST was visible in all wild-type and mutant animals. At the pyramidal decussation, corticospinal axons of all wild-type and mutant mice turned dorsally, crossed the midline and entered the dorsal column of the contralateral side. The sizes of the CST in the medulla and at the pyramidal decussation were comparable in all animals. No pronounced pathfinding errors at the pyramidal decussation were detectable. Analysis of the dorsal columns revealed no marked differences in size or fasciculation.

To evaluate whether any pathfinding errors are detectable just at the time of axonal arrival at the pyramidal decussation, or whether axons reach the decussation with a temporal delay in the mutant animals as it was found e.g. in NCAM deficient mice (Rolf et al., 2002) , we performed a second series of tracing experiments. DiI was applied at postnatal day zero or one for Sema3a and CD24 animals respectively, and animals were analyzed one or two days later to determine whether the formation of the CST is delayed or aberrant in the absence of Sema3A or CD24 (Fig. 5-7). For this investigation we used brains of 7 Sema3A deficient and 17 wild-type littermates and 5 CD24-/- and 7 CD24+/+ animals.

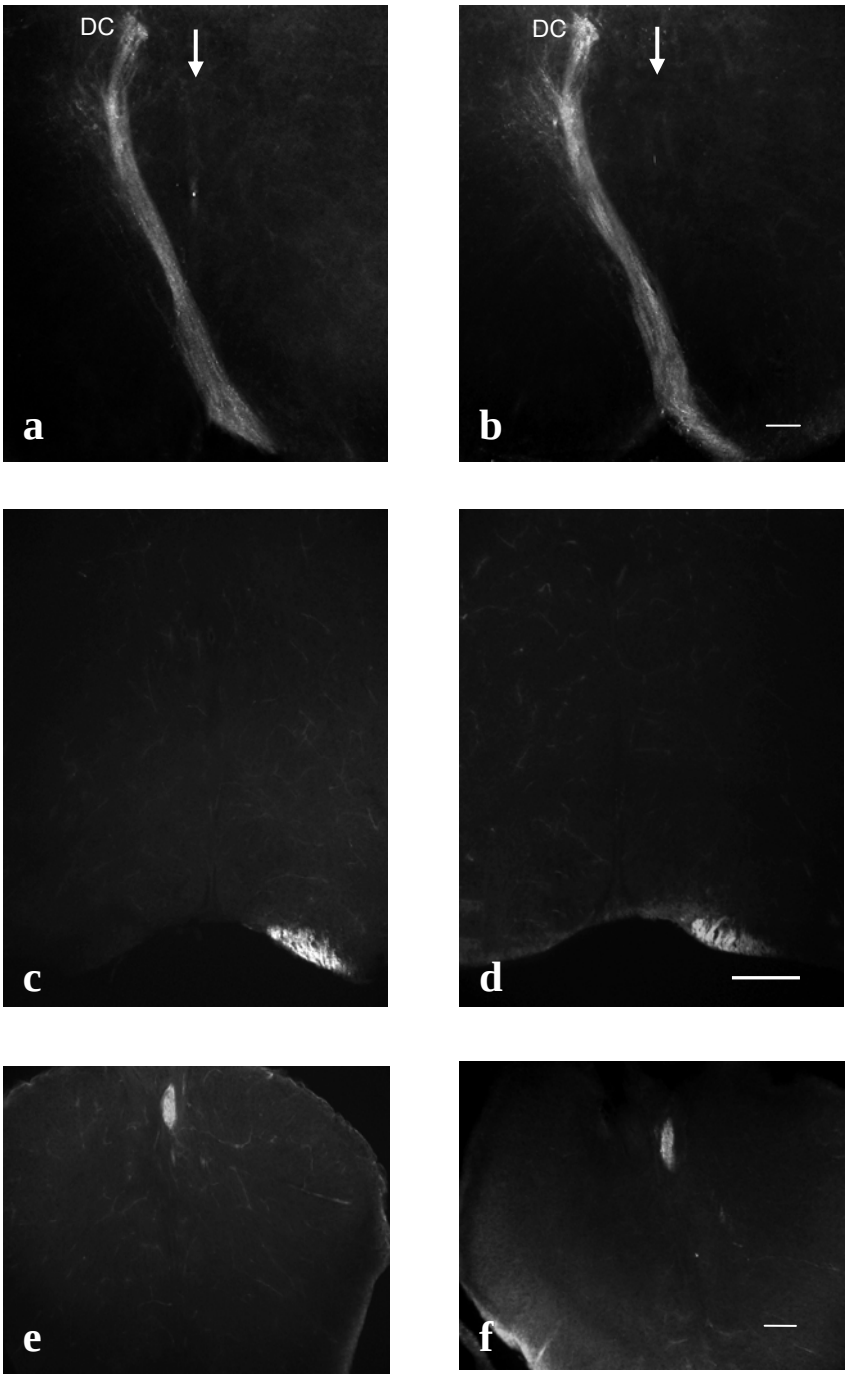


Fig. 5 CST of CD24 mutants
Anterogradly traced CST from P2 homozygous CD24 deficient (right column) and wild-type littermates (left column). Coronal sections.
a,b) Corticospinal axons of two days old wild-type and CD24 mutant mice have crossed the midline (arrows) and entered the contralateral dorsal column (DC).
c,d) Sections more rostral to **a+b**. The size of the pyramidal tract arriving at the pyramidal decussation is similar in CD24 mutant and wild-type littermates.
e,f) Sections more caudal to **a+b** show the dorsal funiculus with a comparable size and fasciculation in CD24-/- and CD24+/+ animals. In all pictures ventral is to the bottom and dorsal is to the top.
Scale bars 200 µm, also valid for the corresponding left picture.

Analysis of the CST at the pyramidal decussation in one / two days old CD24 and Sema3A wild-type and mutant mice revealed the presence of a prominent axon tract that had crossed to the contralateral side and entered the dorsal column (Fig. 5a,b and 7a,b). The size of the CST in the medulla and at the pyramidal decussation was comparable in all animals. No pronounced pathfinding errors at the pyramidal decussation were detectable. Analysis of the ventral anterior CST (Fig. 5c,d) and the dorsal columns (Fig. 5e,f and 7c,d) revealed no differences in size or fasciculation. Since CD24 is located at the midline and could be important for the correct crossing of the midline, this area was thoroughly investigated. Some axons can be found that leave the tract at the ventral midline and apparently do not cross to the contralateral side. This can be seen in mutant as well as in wild-type animals (Fig. 6c,d). On the dorsal contralateral side the tract arborizes in order to innervate interneurons on rostral levels of the spinal cord. The grade of these elaborations also seemed to be comparable in mutant and wild-type animals. The first 'escaping' axons can be found on a similar level of the decussation (Fig. 6a,b). In conclusion the CSTs of Sema3A and CD24 deficient animals show no abnormalities, neither in size, fasciculation, pathfinding nor in temporal development (Table 3).

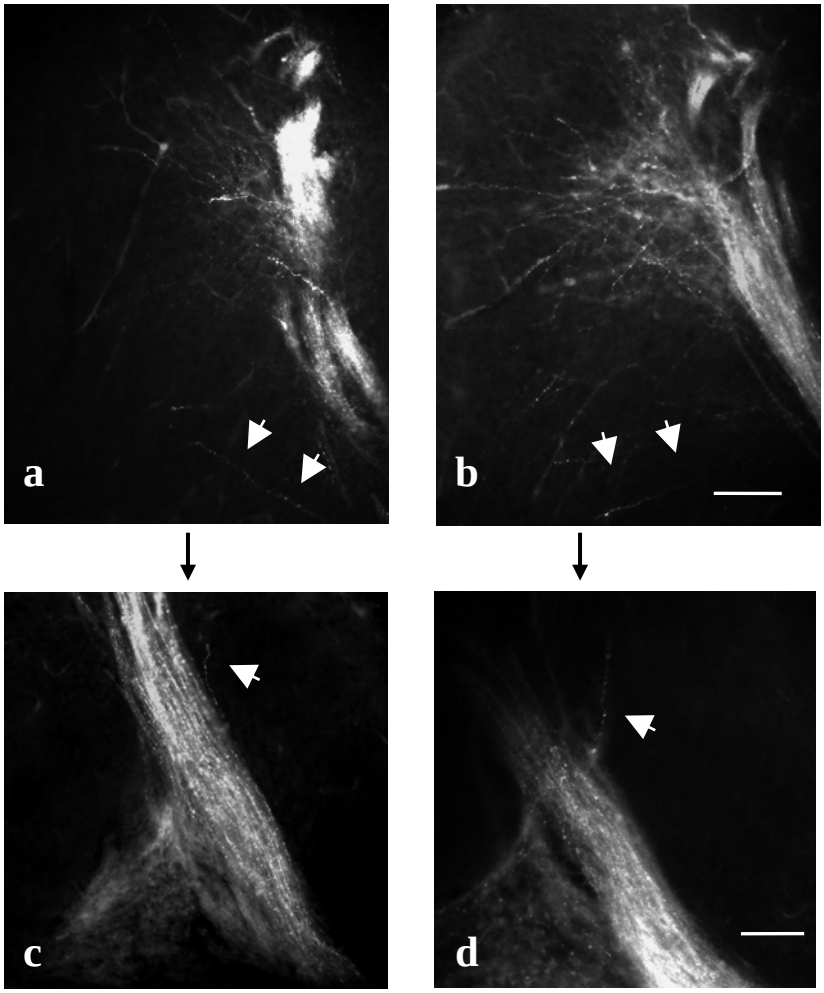


Fig. 6 Pyramidal decussation of CD24 mutants at higher magnifications Anterogradly traced CST from P2 homozygous CD24 deficient (right column) and wild-type littermates (left column). Coronal sections.
a,b) Dorsal elaborations of the CST. The first collaterals growing laterally on the contralateral side leave the tract at comparable ventral levels (arrows).
c,d) Ventral part of the CST. A few axons leave the tract to stay ipsilateral (arrows) before the tract crosses the midline (black arrows). Such fibers could be detected in CD24-/- and CD24+/+ animals.
Scale bars upper row 200 µm, lower row 100 µm, valid also for the corresponding left picture.

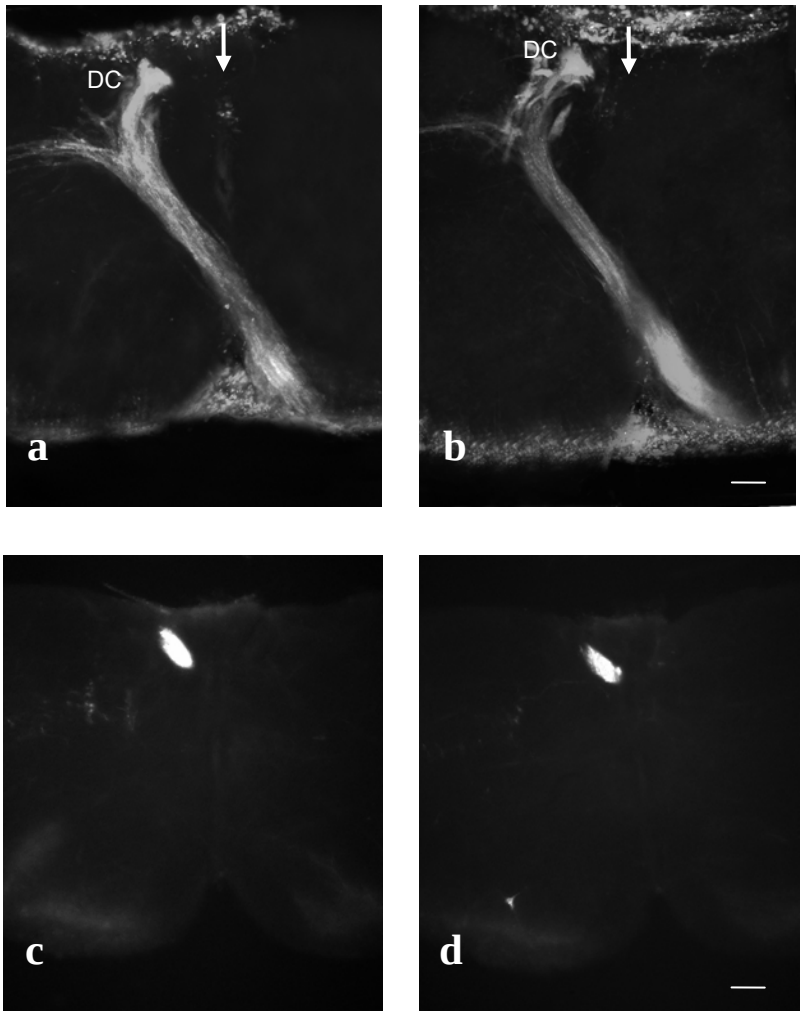


Fig. 7 CST of Sema3A mutants
Anterogradly traced CST of P1 Sema3A homozygous deficient (right column) and wild-type littermates (left column). Coronal sections.
a,b) The CST runs from the ventral right side (bottom right) to the dorsal left side (upper left), thereby crossing the spinal cord midline (arrows). Decussation and dorsalization appear normal in Sema3A mutant mice. DC: dorsal column
c,d) Sections more caudal to **a+b**. The dorsal funiculus is normal in size and fasciculation in Sema3A-/- animals as compared to Sema3A+/+ littermates.
Scale bars 200 µm, valid also for the corresponding left picture.

At E18 the first corticospinal axons just enter the cerebral peduncle overlying the rostral part of the basilar pons. At birth most axons have passed the pons and reach the pyramidal decussation as described above. At that time the first collateral branches become visible on axonal segments overlying the caudal part of the basilar pons. One day later the collateral branches growing into the caudal parts of the pons are well elaborated whereas new collaterals innervating its rostral parts just appear. At P2-3 the mature pattern, characterized by long collaterals into the rostral and caudal parts of the basilar pons, emerges. Both sets of collateral branches are clearly distinct from each

other. The development of the pontine CST shows an alteration in mutant mice expressing L1 ectopically on astrocytes. Here the collateral branches innervated the basilar pons at earlier stages and appeared more diffuse than in wild-type littermates (Ourednik et al., 2001). This finding caused us to examine the pontine collaterals in P1 / P2 Sema3A and CD24 mutant mice, respectively (Fig. 8).

In both wild-type and mutant mice the trajectory of the CST overlaying the basilar pons appeared to be normal. Collateral branches had entered the rostral and caudal portion of the basilar pons in a similar expansion and started out as two distinct bundles in wild-type as well as in mutant mice (Fig. 8).

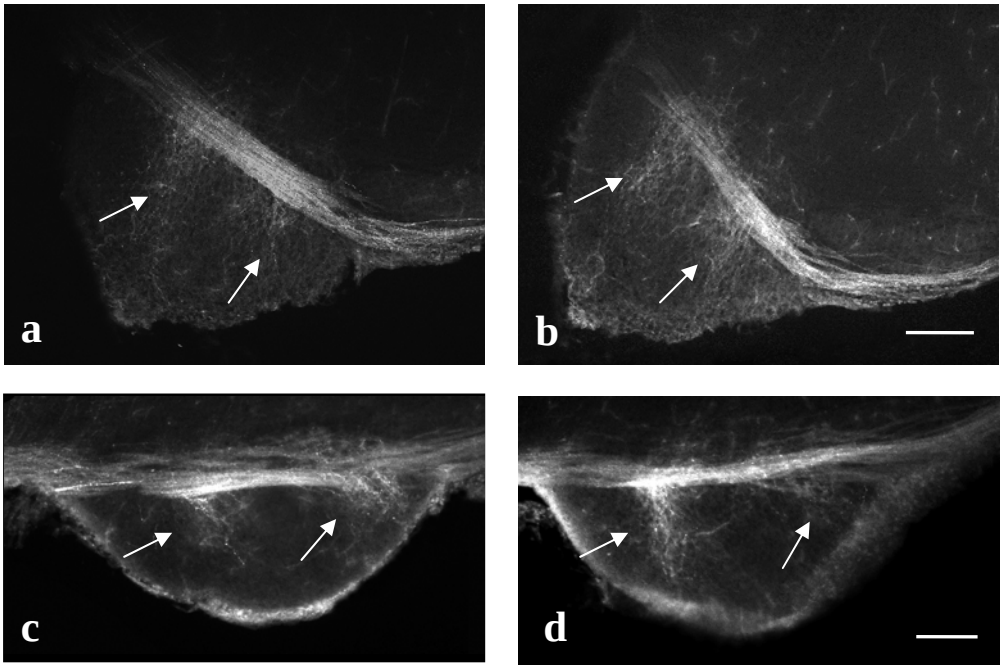


Fig. 8 Sagittal section of the basilar pons of P2 CD24 wild-type (a) and mutant (b) mice and P1 Sema3A wild-type (c) and mutant (d) mice.
The trajectory of the CST overlaying the basilar pons is normal. Collaterals entering the rostral and caudal regions set off as a bundle distinct from each other (arrows). Scale bars 200 µm, also valid for the corresponding left picture.

Investigating the early development of the CST in Sema3A and CD24 deficient mutant mice by anterograde tracings of living animals we did not observe any apparent aberrations

in pathfinding, timing or fasciculation of the tract. In mutant mice size and fasciculation of the tract that arrives at the pyramidal decussation appeared unobtrusive. At the pyramidal decussation axons normally crossed the midline and entered the dorsal column, which was indistinguishable from the wild-type animals. Furthermore, the pontine collateral branches of the CST were normally developed in these animals (Table 3).

	Tracing	**Number**	**Dorsali-zation**	**Cross. of midline**	**Aberrant axons**	**Size dorsal fun.**	**Size cross. tract**	**Pontine fibers**
Sema3A	P1 to P5	3ko 3wt	Normal	Normal	no	Normal	Normal	Normal
Sema3A	P0 to P1	7ko 17wt	Normal	Normal	no	Normal	Normal	Normal
CD24	P1 to P4/5	11ko 17wt	Normal	Normal	no	Normal	Normal	Normal
CD24	P1 to P2	5ko 7wt	Normal	Normal	no	Normal	Normal	Normal

Table 3 Summary of the results from the anterogradly traced CST in Sema3A and CD24 deficient mice

5.0. Discussion

Mice lacking the molecule L1 show among other severe abnormalities pronounced path finding errors combined with a hypoplasia of the CST. Several lines of evidence suggest an interaction between the secreted Sema3A and the surface-linked glycoprotein CD24 that caused us to investigate possible developmental deficits in the CST in mice deficient for these molecules. An investigation of the pyramidal decussation in eight days old mice, an age at that the tract has already fully developed its extension, could not reveal any alterations in Sema3A and CD24 mutant mice. In comparison, pathfinding errors in the L1 deficient mice persisted into the adult animals (Cohen et al., 1998). Elimination of misrouted axons is an often and well organized mechanism in order to establish a fully functional nervous system. Such correction mechanisms could also have taken place in this case as it is suggested for peripheral projections in the Sema3A deficient mouse. Misprojections observed in embryonic mice were all corrected or eliminated by E15.5 (White and Behar, 2000). In mice deficient for the neural cell adhesion molecule NCAM a hypoplasia of the CST persisted in the adult whereas pathfinding errors that were observed in young animals had been eliminated at this stage. Investigations of the early postnatal NCAM mutant mice could not only demonstrate the aberrant growing axons but also revealed a delayed development of the tract (Rolf et al. 2002). In young Sema3A and CD24 mice aged P1 to P4 the CST appeared to be normal. Corticospinal axons decussated to the contralateral side, grew to

the dorsal side and ran down the spinal cord in the dorsal column. Size and fasciculation of the CST were comparable in homozygous mutant mice and wild-type littermates. No misprojections of corticospinal axons or aberrations of the CST were detectable. Some apparently escaping axons that stayed ipsilateral were found in mutant as well as in wild-type littermates representing a described picture as a small percentage of 1 % to 3 % of axons do not cross to the contralateral side in rodents (Reinoso and Castro, 1989). In addition, fibers that leave the tract very early to extend laterally were found in both genotypes and at comparable levels. An analysis of CST collateral branches entering the basilar pons revealed no differences between Sema3A and CD24 deficient mice and their wild-type littermates. In comparison, ectopic L1 expression in astrocytes led to alterations in this region (Ourednik et al., 2002).

Sema3A, highly expressed in the spinal cord, exhibited a strong repulsive effect on cortical axons *in vitro* (Castellani et al., 2002); making it highly suggestive that Sema3A is an important player guiding corticospinal axons by interacting with L1 via its receptor neuropilin-1. However, its deficiency had no detectable consequences on the development of the long projecting CST. Since the Sema3A deficient mouse showed morphological defects in the peripheral nervous system (Taniguchi et al., 1997), we think that the Sema3A function is indeed severely comprised in these animals and that not a still sufficient protein level limits the pathfinding phenotype. Our results suggest that Sema3A is not essential on its own for the formation of the CST. The semaphorins are a large group of secreted and transmembrane proteins with partially overlapping expression patterns in the developing CNS. E.g. Sema3C is expressed in the ventral spinal cord partly additionally to Sema3A (Püschel et al., 1996). Other family members whose expressions were described for the CNS include Sema6D (Qu et al., 2002; Taniguchi et al., 2004), Sema4D (Moreau-Fauvarque et al., 2003) and Sema3G (Li et al., 1999). They all inherit a potential to compensate for the lack of Sema3A. Furthermore, the family of semaphorins is still growing with new members being steadily identified (Qu et al., 2002; Moreau-Fauvarque et al., 2003). Thus, more prospective candidates may soon appear that will be worth exploring in respect of motoaxon guidance and in particular the guidance of the CST. Other guidance cues or their receptors such as netrins, ephrins or CAMs may also be involved in feedback regulations enabling proper development of a ‘resilient’ CST.

As the peripheral nervous system of the Sema3A deficient mouse shows severe developmental defects in both independently generated mouse lines, such redundancy mechanisms might be in particularly existent in the central nervous system. This is supported by a study on the Sema3A deficient mouse generated by Behar et al. (Catalano et al., 1998) that found a normal development of many major axonal projections, among others cerebellar climbing fibers and basal forebrain projections. In addition, dorsal root ganglion afferents in the spinal cord appeared normal in the second mutant line (Taniguchi et al., 1997). Given the *in vitro* functional analysis one would have expected severe abnormalities for certain classes of sensory axons (Messersmith et al., 1995). Similarly, mice deficient for the Sema3A receptor neuropilin-1 exhibited disruptions of the peripheral nerve projections comparable with the Sema3A mutant mouse. On the contrary, the afferent sensory projections appeared normal and so far no deficits in the CNS have been described (Kitsukawa et al., 1997). Since neuropilin-1 mutants die at E12.5 prior to a developmental stage where differential sensory afferent innervation in the spinal cord can be clearly visualized (the establishment of the sensory afferent termination pattern is just about to begin at E13; Ozaki and Snider, 1997), it still remains open whether neuropilin-1 deficiency causes defects in the dorsal root ganglion sensory afferents within the spinal cord and possibly other CNS projections or not. To verify an *in vivo* function of the Sema3A-neuropilin-1 interaction in the developing CNS as it was suggested by the numerous *in vitro* studies, it may be interesting to see whether neuropilin-1 heterozygous animals in combination with Sema3A deficiency show abnormalities. A similar experiment has been performed in zebrafish. Here the lack of Sema3A that by itself did not lead to a phenotype, in addition to a reduced level of neuropilin-1, subthreshold to effects caused by solely neuropilin-1 deficiency, led to pathfinding errors of motoaxons (Feldner et al., 2005).

The importance of CD24 in the nervous system has only rarely been investigated. Its deficiency at the subventricular zone leads to alterations of neurogenesis (Belvindrah et al., 2002; Nieoullon et al., 2005). Which role does it play at the midline of the pyramidal decussation? It could well be that also here redundancy plays a role. Genetic analysis of other guidance molecules has led to similar conclusions. For example, netrin-1 has been shown to exhibit repulsive actions on trochlear axons *in vitro* but *in vivo* analysis of a deficient mouse could not reveal such a phenotype (Serafini et al.,

1994, 1996). Similarly, loss-of function mutations in Sema2a did not generate detectable abnormalities in the Drosophila CNS (Kolodkin et al., 1993). Another possibility is that the expression of CD24 at the midline had its importance at other developmental stages. The different glycoforms of CD24 seem to play an important role in its functions. Different cell types have been shown to exhibit different glycosylations (Kleene et al., 2001). The binding onto L1 crucially depends on sialic acid and the sugar LewisX. Whether these glycosylations are found on CD24 expressed at the midline is currently unknown. Several *in vitro* studies using cerebellar and dorsal root ganglion neurons show L1 dependent effects of CD24 on neurite outgrowth (Shewan et al. 1996; Kleene et al. 2001). To investigate whether a similar effect can be found using motoneurons or cortical explants will help to understand the role of CD24 at the pyramidal decussation. In addition it will be interesting to investigate projections that are suggested by the *in vitro* data such as the dorsal root ganglia afferents and efferents. Such investigations will elucidate CD24's role in axonal guidance also in respect to a possible interplay with Sema3A and L1.

5.1. Conclusion

In conclusion we propose that the lack of axonal phenotype in Sema3A and CD24 mutant mice may be due to the presence of other related or non-related guidance molecules and receptors. Important guidance decisions during the formation of the nervous system do not solely depend on the expression of a single guidance cue. Though L1 deficient mice show severe abnormalities the defects are more subtle than one could have predicted from its expression pattern (Cohen et al. 1998). Various cues that exhibit differentiated actions dependent on varying glycosylations and the display of certain peptide sequences, an interaction with particular ligands, receptors or coreceptors, the state of activity of the cell and temporal and spatial regulated expression patterns, act in concert to ensure a robust and reliable formation of important pathways. The erasure of one of these components often seems to be dispensable. Under this light the generation of double and triple mutant mice may represent a fruitful genetic approach to dissect the mechanisms that control the formation of axon pathways in the developing brain.

Bibliography

Unified nomenclature for the semaphorins/collapsins. Semaphorin Nomenclature Committee. *Cell* 97 (5):551-552, 1999.

R. H. Adams, H. Betz, and A. W. Puschel. A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis. *Mech.Dev.* 57 (1):33-45, 1996.

C. B. Aguiar, B. Lobao-Soares, M. Alvarez-Silva, and A. G. Trentin . Glycosaminoglycans modulate C6 glioma cell adhesion to extracellular matrix components and alter cell proliferation and cell migration . *BMC.Cell Biol.* 6:31, 2005.

J. Altman and G. D. Das. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J.Comp Neurol.* 124 (3):319-335, 1965.

J. Altman. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J.Comp Neurol.* 137 (4):433-457, 1969.

D. N. Angelov, M. Walther, M. Streppel, O. Guntinas-Lichius, W. F. Neiss, R. Probstmeier, and P. Pesheva. Tenascin-R is antiadhesive for activated microglia that induce downregulation of the protein after peripheral nerve injury: a new role in neuronal protection. *J.Neurosci.* 18 (16):6218-6229, 1998.

E. Aufderheide, R. Chiquet-Ehrismann, and P. Ekblom. Epithelial-mesenchymal interactions in the developing kidney lead to expression of tenascin in the mesenchyme. *J.Cell Biol.* 105 (1):599-608, 1987.

E. Aufderheide and P. Ekblom. Tenascin during gut development: appearance in the mesenchyme, shift in molecular forms, and dependence on epithelial-mesenchymal interactions. *J.Cell Biol.* 107 (6 Pt 1):2341-2349, 1988.

J. R. Backstrom, G. P. Lim, M. J. Cullen, and Z. A. Tokes. Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40). *J.Neurosci.* 16 (24):7910-7919, 1996.

C. E. Bandtlow and D. R. Zimmermann. Proteoglycans in the developing brain: new conceptual insights for old proteins. *Physiol Rev.* 80 (4):1267-1290, 2000.

S. Bartsch, U. Bartsch, U. Dorries, A. Faissner, A. Weller, P. Ekblom, and M. Schachner. Expression of tenascin in the developing and adult cerebellar cortex. *J.Neurosci.* 12 (3):736-749, 1992.

S. Bartsch, K. Husmann, M. Schachner, and U. Bartsch. The extracellular matrix molecule tenascin: expression in the developing chick retinotectal system and substrate properties for retinal ganglion cell neurites in vitro. *Eur.J.Neurosci.* 7 (5):907-916, 1995.

U. Bartsch, P. Pesheva, M. Raff, and M. Schachner. Expression of janusin (J1-160/180) in the retina and optic nerve of the developing and adult mouse. *Glia* 9 (1):57-69, 1993.

U. Bartsch, A. Faissner, J. Trotter, U. Dorries, S. Bartsch, H. Mohajeri, and M. Schachner. Tenascin demarcates the boundary between the myelinated and nonmyelinated part of retinal ganglion cell axons in the developing and adult mouse. *J.Neurosci.* 14 (8):4756-4768, 1994.

U. Bartsch. The extracellular matrix molecule tenascin-C: expression in vivo and functional characterization in vitro. *Prog.Neurobiol.* 49 (2):145-168, 1996.

M. Bastmeyer and D. D. O'Leary. Dynamics of target recognition by interstitial axon branching along developing cortical axons. *J.Neurosci.* 16 (4):1450-1459, 1996.

C. G. Becker, T. Becker, R. L. Meyer, and M. Schachner. Tenascin-R inhibits the growth of optic fibers in vitro but is rapidly eliminated during nerve regeneration in the salamander Pleurodeles waltl . *J.Neurosci.* 19 (2):813-827, 1999.

C. G. Becker, J. Schweitzer, J. Feldner, T. Becker, and M. Schachner. Tenascin-R as a repellent guidance molecule for developing optic axons in zebrafish. *J.Neurosci.* 23 (15):6232-6237, 2003.

T. Becker, B. Anliker, C. G. Becker, J. Taylor, M. Schachner, R. L. Meyer, and U. Bartsch. Tenascin-R inhibits regrowth of optic fibers in vitro and persists in the optic nerve of mice after injury. *Glia* 29 (4):330-346, 2000.

O. Behar, J. A. Golden, H. Mashimo, F. J. Schoen, and M. C. Fishman. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature* 383 (6600):525-528, 1996.

R. Belvindrah, G. Rougon, and G. Chazal. Increased neurogenesis in adult mCD24-deficient mice. *J.Neurosci.* 22 (9):3594-3607, 2002.

F. Benninger, H. Beck, M. Wernig, K. L. Tucker, O. Brustle, and B. Scheffler. Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. *J.Neurosci.* 23 (18):7075-7083, 2003.

A. J. Bieber, P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell* 59 (3):447-460, 1989.

L. M. Bjorklund, R. Sanchez-Pernaute, S. Chung, T. Andersson, I. Y. Chen, K. S. McNaught, A. L. Brownell, B. G. Jenkins, C. Wahlestedt, K. S. Kim, and O. Isacson. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc.Natl.Acad.Sci.U.S.A* 99 (4):2344-2349, 2002.

C. R. Bjornson, R. L. Rietze, B. A. Reynolds, M. C. Magli, and A. L. Vescovi. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 283 (5401):534-537, 1999.

C. V. Borlongan. Transplantation therapy for Parkinson's disease. *Expert.Opin.Investig.Drugs* 9 (10):2319-2330, 2000.

M. A. Bourdon, C. J. Wikstrand, H. Furthmayr, T. J. Matthews, and D. D. Bigner. Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. *Cancer Res.* 43 (6):2796-2805, 1983.

F. Brenneke, M. Schachner, C. E. Elger, and A. A. Lie. Up-regulation of the extracellular matrix glycoprotein tenascin-R during axonal reorganization and astrogliosis in the adult rat hippocampus. *Epilepsy Res.* 58 (2-3):133-143, 2004.

J. Bristow, M. K. Tee, S. E. Gitelman, S. H. Mellon, and W. L. Miller. Tenascin-X: a novel extracellular matrix protein encoded by the human XB gene overlapping P450c21B. *J.Cell Biol.* 122 (1):265-278, 1993.

M. Brown. Essentials of neural development. Anonymous. Anonymous. *Cambridge University Press* , 1991.

G. Bruckner, J. Grosche, S. Schmidt, W. Hartig, R. U. Margolis, B. Delpech, C. I. Seidenbecher, R. Czaniera, and M. Schachner. Postnatal development of perineuronal nets in wild-type mice and in a mutant deficient in tenascin-R. *J.Comp Neurol.* 428 (4):616-629, 2000.

T. Brummendorf and F. G. Rathjen. Axonal glycoproteins with immunoglobulin- and fibronectin type III-related domains in vertebrates: structural features, binding activities, and signal transduction. *J.Neurochem.* 61 (4):1207-1219, 1993.

T. Brummendorf and F. G. Rathjen. Structure/function relationships of axon-associated adhesion receptors of the immunoglobulin superfamily. *Curr.Opin.Neurobiol.* 6 (5):584-593, 1996.

T. Brummendorf, S. Kenwrick, and F. G. Rathjen. Neural cell recognition molecule L1: from cell biology to human hereditary brain malformations. *Curr.Opin.Neurobiol.* 8 (1):87-97, 1998.

O. Brustle, A. C. Spiro, K. Karram, K. Choudhary, S. Okabe, and R. D. McKay. In vitro-generated neural precursors participate in mammalian brain development. *Proc.Natl.Acad.Sci.U.S.A* 94 (26):14809-14814, 1997.

S. M. Burden-Gulley and V. Lemmon. L1, N-cadherin, and laminin induce distinct distribution patterns of cytoskeletal elements in growth cones. *Cell Motil.Cytoskeleton* 35 (1):1-23, 1996.

S. M. Burden-Gulley, M. Pendergast , and V. Lemmon. The role of cell adhesion molecule L1 in axonal extension, growth cone motility, and signal transduction. *Cell Tissue Res.* 290 (2):415-422, 1997.

R. C. Burrows, D. Wancio, P. Levitt, and L. Lillien. Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron* 19 (2):251-267, 1997.

D. V. Caccamo, C. D. Katsetos, A. Frankfurter, V. P. Collins, S. R. Vandenburg, and M. M. Herman. An immunohistochemical characterization of the primitive and maturing neuroepithelial components in the OTT-6050 transplantable mouse teratoma. *Neuropathol.Appl.Neurobiol.* 15 (5):389-405, 1989.

H. A. Cameron, C. S. Woolley, B. S. McEwen, and E. Gould. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56 (2):337-344, 1993.

H. A. Cameron, T. G. Hazel, and R. D. McKay. Regulation of neurogenesis by growth factors and neurotransmitters. *J.Neurobiol.* 36 (2):287-306, 1998.

D. Caric, H. Raphael, J. Viti, A. Feathers, D. Wancio, and L. Lillien. EGFRs mediate chemotactic migration in the developing telencephalon. *Development* 128 (21):4203-4216, 2001.

V. Castellani, A. Chedotal, M. Schachner, C. Faivre-Sarrailh, and G. Rougon. Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* 27 (2):237-249, 2000.

S. M. Catalano, E. K. Messersmith, C. S. Goodman, C. J. Shatz, and A. Chedotal. Many major CNS axon projections develop normally in the absence of semaphorin III. *Mol.Cell Neurosci.* 11 (4):173-182, 1998.

M. R. Celio and R. Chiquet-Ehrismann. 'Perineuronal nets' around cortical interneurons expressing parvalbumin are rich in tenascin. *Neurosci.Lett.* 162 (1-2):137-140, 1993.

A. Chedotal, J. A. Del Rio, M. Ruiz, Z. He, V. Borrell, F. de Castro, F. Ezan, C. S. Goodman, M. Tessier-Lavigne, C. Sotelo, and E. Soriano. Semaphorins III and IV repel hippocampal axons via two distinct receptors. *Development* 125 (21):4313-4323, 1998.

H. Chen, Z. He, and M. Tessier-Lavigne. Axon guidance mechanisms: semaphorins as simultaneous repellents and anti-repellents. *Nat.Neurosci.* 1 (6):436-439, 1998.

R. Chiquet-Ehrismann, E. J. Mackie , C. A. Pearson, and T. Sakakura . Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* 47 (1):131-139, 1986.

R. Chiquet-Ehrismann, C. Hagios, and K. Matsumoto. The tenascin gene family. *Perspect.Dev.Neurobiol.* 2 (1):3-7, 1994.

R. Chiquet-Ehrismann and R. P. Tucker. Connective tissues: signalling by tenascins. *Int.J.Biochem.Cell Biol.* 36 (6):1085-1089, 2004.

R. Chiquet-Ehrismann. Tenascins. *Int.J.Biochem.Cell Biol.* 36 (6):986-990, 2004.

M. Chiquet and D. M. Fambrough. Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunits. *J.Cell Biol.* 98 (6):1937-1946, 1984.

M. Chiquet and B. Wehrle-Haller. Tenascin-C in peripheral nerve morphogenesis. *Perspect.Dev.Neurobiol.* 2 (1):67-74, 1994.

C. Y. Chung and H. P. Erickson. Cell surface annexin II is a high affinity receptor for the alternatively spliced segment of tenascin-C. *J.Cell Biol.* 126 (2):539-548, 1994.

C. Y. Chung, J. E. Murphy-Ullrich, and H. P. Erickson. Mitogenesis, cell migration, and loss of focal adhesions induced by tenascin-C interacting with its cell surface receptor, annexin II. *Mol.Biol.Cell* 7 (6):883-892, 1996.

C. Cifuentes-Diaz, E. Velasco, F. A. Meunier, D. Goudou, L. Belkadi, L. Faille, M. Murawsky, D. Angaut-Petit, J. Molgo, M. Schachner, Y. Saga, S. Aizawa, and F. Rieger. The peripheral nerve and the neuromuscular junction are affected in the tenascin-C-deficient mouse. *Cell Mol.Biol.(Noisy.-le-grand)* 44 (2):357-379, 1998.

N. R. Cohen, J. S. Taylor, L. B. Scott, R. W. Guillery, P. Soriano, and A. J. Furley. Errors in corticospinal axon guidance in mice lacking the neural cell adhesion molecule L1. *Curr.Biol.* 8 (1):26-33, 1998.

R. I. Cohen, D. M. Rottkamp, D. Maric, J. L. Barker, and L. D. Hudson. A role for semaphorins and neuropilins in oligodendrocyte guidance. *J.Neurochem.* 85 (5):1262-1278, 2003.

J. F. Conway and D. A. Parry. Three-stranded alpha-fibrous proteins: the heptad repeat and its implications for structure. *Int.J.Biol.Macromol.* 13 (1):14-16, 1991.

R. M. Cooke, A. J. Wilkinson, M. Baron, A. Pastore, M. J. Tappin, I. D. Campbell, H. Gregory, and B. Sheard. The solution structure of human epidermal growth factor. *Nature* 327 (6120):339-341, 1987.

F. S. Corotto, J. A. Henegar, and J. A. Maruniak. Neurogenesis persists in the subependymal layer of

the adult mouse brain. *Neurosci.Lett.* 149 (2):111-114, 1993.

K. L. Crossin, S. Hoffman, M. Grumet, J. P. Thiery, and G. M. Edelman. Site-restricted expression of cytotactin during development of the chicken embryo. *J.Cell Biol.* 102 (5):1917-1930, 1986.

K. L. Crossin, S. Hoffman, S. S. Tan, and G. M. Edelman. Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex of the early postnatal mouse. *Dev.Biol.* 136 (2):381-392, 1989.

K. L. Crossin, A. L. Prieto, S. Hoffman, F. S. Jones, and D. R. Friedlander. Expression of adhesion molecules and the establishment of boundaries during embryonic and neural development. *Exp.Neurol.* 109 (1):6-18, 1990.

L. D'Alessandri, B. Ranscht, K. H. Winterhalter, and L. Vaughan. Contactin/F11 and tenascin-C co-expression in the chick retina correlates with formation of the synaptic plexiform layers. *Curr.Eye Res.* 14 (10):911-926, 1995.

M. Dahme, U. Bartsch, R. Martini, B. Anliker, M. Schachner, and N. Mantei. Disruption of the mouse L1 gene leads to malformations of the nervous system. *Nat.Genet.* 17 (3):346-349, 1997.

G. D. Das. Gliogenesis during embryonic development in the rat. *Experientia* 33 (12):1648-1649, 1977.

A. M. Davies. Neurotrophins: neurotrophic modulation of neurite growth. *Curr.Biol.* 10 (5):R198-R200, 2000.

A. A. Davis and S. Temple. A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* 372 (6503):263-266, 1994.

J. Q. Davis and V. Bennett. Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. *J.Biol.Chem.* 269 (44):27163-27166, 1994.

M. S. Deiner and D. W. Sretavan. Altered midline axon pathways and ectopic neurons in the developing hypothalamus of netrin-1- and DCC-deficient mice. *J.Neurosci.* 19 (22):9900-9912, 1999.

G. P. Demyanenko, A. Y. Tsai, and P. F. Maness. Abnormalities in neuronal process extension, hippocampal development, and the ventricular system of L1 knockout mice. *J.Neurosci.* 19 (12):4907-4920, 1999.

M. Dihne, C. Bernreuther, M. Sibbe, W. Paulus, and M. Schachner. A new role for the cell adhesion molecule L1 in neural precursor cell proliferation, differentiation, and transmitter-specific subtype generation. *J.Neurosci.* 23 (16):6638-6650, 2003.

P. J. Donovan and J. Gearhart. The end of the beginning for pluripotent stem cells. *Nature* 414 (6859):92-97, 2001.

U. Dorries and M. Schachner. Tenascin mRNA isoforms in the developing mouse brain. *J.Neurosci.Res.* 37 (3):336-347, 1994.

U. Dorries, J. Taylor, Z. Xiao, A. Lochter, D. Montag, and M. Schachner. Distinct effects of recombinant tenascin-C domains on neuronal cell adhesion, growth cone guidance, and neuronal polarity. *J.Neurosci.Res.* 43 (4):420-438, 1996.

R. Drucker-Colin and L. Verdugo-Diaz. Cell transplantation for Parkinson's disease: present status. *Cell Mol.Neurobiol.* 24 (3):301-316, 2004.

Y. Echelard, D. J. Epstein, B. St Jacques, L. Shen, J. Mohler, J. A. McMahon, and A. P. McMahon. Sonic hedgehog, a member of a family of putative

signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75 (7):1417-1430, 1993.

F. Elefteriou, J. Y. Exposito, R. Garrone, and C. Lethias. Characterization of the bovine tenascin-X. *J.Biol.Chem.* 272 (36):22866-22874, 1997.

U. Englund, A. Bjorklund, and K. Wictorin. Migration patterns and phenotypic differentiation of long-term expanded human neural progenitor cells after transplantation into the adult rat brain. *Brain Res.Dev.Brain Res.* 134 (1-2):123-141, 2002.

H. P. Erickson and J. L. Inglesias . A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature* 311 (5983):267-269, 1984.

H. P. Erickson. Evolution of the tenascin family--implications for function of the C-terminal fibrinogen-like domain. *Perspect.Dev.Neurobiol.* 2 (1):9-19, 1994.

M. R. Evers, B. Salmen, O. Bukalo, A. Rollenhagen, M. R. Bosl, F. Morellini, U. Bartsch, A. Dityatev, and M. Schachner. Impairment of L-type Ca2+ channel-dependent forms of hippocampal synaptic plasticity in mice deficient in the extracellular matrix glycoprotein tenascin-C. *J.Neurosci.* 22 (16):7177-7194, 2002.

A. Faissner and J. Kruse. J1/tenascin is a repulsive substrate for central nervous system neurons. *Neuron* 5 (5):627-637, 1990.

A. Faissner. Tenascin glycoproteins in neural pattern formation: facets of a complex picture. *Perspect.Dev.Neurobiol.* 1 (3):155-164, 1993.

J. Fan, S. G. Mansfield, T. Redmond, P. R. Gordon-Weeks, and J. A. Raper. The organization of F-actin and microtubules in growth cones exposed to a brain-derived collapsing factor. *J.Cell Biol.* 121 (4):867-878, 1993.

J. Fan and J. A. Raper. Localized collapsing cues can steer growth cones without inducing their full collapse. *Neuron* 14 (2):263-274, 1995.

J. Feldner, T. Becker, K. Goishi, J. Schweitzer, P. Lee, M. Schachner, M. Klagsbrun, and C. G. Becker. Neuropilin-1a is involved in trunk motor axon outgrowth in embryonic zebrafish. *Dev.Dyn.* 234 (3):535-549, 2005.

L. Ferhat, Louis N. Chevassus au, I. Jorquera, J. Niquet, M. Khrestchatisky, Y. Ben Ari, and A. Represa. Transient increase of tenascin-C in immature hippocampus: astroglial and neuronal expression. *J.Neurocytol.* 25 (1):53-66, 1996.

G. F. Fischer, O. Majdic, S. Gadd, and W. Knapp. Signal transduction in lymphocytic and myeloid cells via CD24, a new member of phosphoinositol-anchored membrane molecules. *J.Immunol.* 144 (2):638-641, 1990.

M. Fluck, V. Tunc-Civelek, and M. Chiquet. Rapid and reciprocal regulation of tenascin-C and tenascin-Y expression by loading of skeletal muscle. *J.Cell Sci.* 113 (Pt 20):3583-3591, 2000.

E. Fransen, V. Lemmon, G. Van Camp, L. Vits, P. Coucke, and P. J. Willems. CRASH syndrome: clinical spectrum of corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraparesis and hydrocephalus due to mutations in one single gene, L1. *Eur.J.Hum.Genet.* 3 (5):273-284, 1995.

E. Fransen, R. D'Hooge, G. Van Camp, M. Verhoye, J. Sijbers, E. Reyniers, P. Soriano, H. Kamiguchi, R. Willemsen, S. K. Koekkoek, C. I. De Zeeuw, P. P. De Deyn, Linden A. Van der, V. Lemmon, R. F. Kooy, and P. J. Willems. L1 knockout mice show dilated ventricles, vermis hypoplasia and impaired exploration patterns. *Hum.Mol.Genet.* 7 (6):999-1009, 1998.

S. Freitag, M. Schachner, and F. Morellini. Behavioral alterations in mice deficient for the extracellular matrix glycoprotein tenascin-R. *Behav.Brain Res.* 145 (1-2):189-207, 2003.

R. A. Fricker, M. K. Carpenter, C. Winkler, C. Greco, M. A. Gates, and A. Bjorklund. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J.Neurosci.* 19 (14):5990-6005, 1999.

E. Frost, B. W. Kiernan, A. Faissner, and C. ffrench-Constant. Regulation of oligodendrocyte precursor migration by extracellular matrix: evidence for substrate-specific inhibition of migration by tenascin-C. *Dev.Neurosci.* 18 (4):266-273, 1996.

S. Y. Fu, K. Sharma, Y. Luo, J. A. Raper, and E. Frank. SEMA3A regulates developing sensory projections in the chicken spinal cord. *J.Neurobiol.* 45 (4):227-236, 2000.

K. E. Fujimori, K. Takeuchi, T. Yazaki, K. Uyemura, Y. Nojyo, and N. Tamamki. Expression of L1 and TAG-1 in the corticospinal, callosal, and hippocampal commissural neurons in the developing rat telencephalon as revealed by retrograde and in situ hybridization double labeling. *J.Comp Neurol.* 417 (3):275-288, 2000.

F. Fukamauchi, N. Mataga, Y. J. Wang, S. Sato, A. Youshiki, and M. Kusakabe. Abnormal behavior and neurotransmissions of tenascin gene knockout mouse. *Biochem.Biophys.Res.Commun.* 221 (1):151-156, 1996.

B. Fuss, U. Pott, P. Fischer, M. E. Schwab, and M. Schachner. Identification of a cDNA clone specific for the oligodendrocyte-derived repulsive extracellular matrix molecule J1-160/180. *J.Neurosci.Res.* 29 (3):299-307, 1991.

B. Fuss, E. S. Wintergerst, U. Bartsch, and M. Schachner. Molecular characterization and in situ mRNA localization of the neural recognition molecule J1-160/180: a modular structure similar to tenascin. *J.Cell Biol.* 120 (5):1237-1249, 1993.

E. Garcion, A. Faissner, and C. ffrench-Constant. Knockout mice reveal a contribution of the extracellular matrix molecule tenascin-C to neural precursor proliferation and migration. *Development* 128 (13):2485-2496, 2001.

E. Garcion, A. Halilagic, A. Faissner, and C. ffrench-Constant. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. *Development* 131 (14):3423-3432, 2004.

J. Garwood, E. Garcion, A. Dobbertin, N. Heck, V. Calco, C. ffrench-Constant, and A. Faissner. The extracellular matrix glycoprotein Tenascin-C is expressed by oligodendrocyte precursor cells and required for the regulation of maturation rate, survival and responsiveness to platelet-derived growth factor. *Eur.J.Neurosci.* 20 (10):2524-2540, 2004.

F. Gomez-Pinilla, D. J. Knauer, and M. Nieto-Sampedro. Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. *Brain Res.* 438 (1-2):385-390, 1988.

M. L. Gonzalez and J. Silver. Axon-glia interactions regulate ECM patterning in the postnatal rat olfactory bulb. *J.Neurosci.* 14 (10):6121-6131, 1994.

C. S. Goodman and C. J. Shatz. Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* 72 Suppl:77-98, 1993.

B. Gotz, A. Scholze, A. Clement, A. Joester, K. Schutte, F. Wigger, R. Frank, E. Spiess, P. Ekblom, and A. Faissner. Tenascin-C contains distinct adhesive, anti-adhesive, and neurite outgrowth promoting sites for neurons. *J.Cell Biol.* 132 (4):681-699, 1996.

E. Gould, A. J. Reeves, M. S. Graziano, and C. G. Gross. Neurogenesis in the neocortex of adult primates. *Science* 286 (5439):548-552, 1999.

A. Gritti, E. A. Parati, L. Cova, P. Frolichsthal, R. Galli, E. Wanke, L. Faravelli, D. J. Morassutti, F. Roisen, D. D. Nickel, and A. L. Vescovi. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J.Neurosci.* 16 (3):1091-1100, 1996.

A. Gritti, P. Frolichsthal-Schoeller, R. Galli, E. A. Parati, L. Cova, S. F. Pagano, C. R. Bjornson, and A. L. Vescovi. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J.Neurosci.* 19 (9):3287-3297, 1999.

R. E. Gross, M. F. Mehler, P. C. Mabie, Z. Zang, L. Santschi, and J. A. Kessler. Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron* 17 (4):595-606, 1996.

M. Grumet, S. Hoffman, and G. M. Edelman. Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron-neuron and neuron-glia adhesion. *Proc.Natl.Acad.Sci.U.S.A* 81 (1):267-271, 1984.

M. Grumet, S. Hoffman, K. L. Crossin, and G. M. Edelman. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc.Natl.Acad.Sci.U.S.A* 82 (23):8075-8079, 1985.

M. Grumet, V. Mauro, M. P. Burgoon, G. M. Edelman, and B. A. Cunningham. Structure of a new nervous system glycoprotein, Nr-CAM, and its relationship to subgroups of neural cell adhesion molecules. *J.Cell Biol.* 113 (6):1399-1412, 1991.

D. Gundersen, C. Tran-Thang, B. Sordat, F. Mourali, and C. Ruegg. Plasmin-induced proteolysis of tenascin-C: modulation by T lymphocyte-derived urokinase-type plasminogen activator and effect on T lymphocyte adhesion, activation, and cell clustering. *J.Immunol.* 158 (3):1051-1060, 1997.

M. A. Hack, M. Sugimori, C. Lundberg, M. Nakafuku, and M. Gotz. Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol.Cell Neurosci.* 25 (4):664-678, 2004.

K. Hagihara, R. Miura, R. Kosaki, E. Berglund, B. Ranscht, and Y. Yamaguchi. Immunohistochemical evidence for the brevican-tenascin-R interaction: colocalization in perineuronal nets suggests a physiological role for the interaction in the adult rat brain. *J.Comp Neurol.* 410 (2):256-264, 1999.

C. Hagios, M. Koch, J. Spring, M. Chiquet, and R. Chiquet-Ehrismann. Tenascin-Y: a protein of novel domain structure is secreted by differentiated fibroblasts of muscle connective tissue. *J.Cell Biol.* 134 (6):1499-1512, 1996.

M. Hahne, R. H. Wenger, D. Vestweber, and P. J. Nielsen. The heat-stable antigen can alter very late antigen 4-mediated adhesion. *J.Exp.Med.* 179 (4):1391-1395, 1994.

W. Halfter, R. Chiquet-Ehrismann, and R. P. Tucker. The effect of tenascin and embryonic basal lamina on the behavior and morphology of neural crest cells in vitro. *Dev.Biol.* 132 (1):14-25, 1989.

K. T. Hall, L. Boumsell, J. L. Schultze, V. A. Boussiotis, D. M. Dorfman, A. A. Cardoso, A. Bensussan, L. M. Nadler, and G. J. Freeman. Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. *Proc.Natl.Acad.Sci.U.S.A* 93 (21):11780-11785, 1996.

Z. He and M. Tessier-Lavigne. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* 90 (4):739-751, 1997.

J. E. Holley, D. Gveric, J. L. Whatmore, and N. J. Gutowski. Tenascin C induces a quiescent phenotype in cultured adult human astrocytes. *Glia*, 2005.

J. Holm, R. Hillenbrand, V. Steuber, U. Bartsch, M. Moos, H. Lubbert, D. Montag, and M. Schachner. Structural features of a close homologue of L1 (CHL1) in the mouse: a new member of the L1 family of neural recognition molecules. *Eur.J.Neurosci.* 8 (8):1613-1629, 1996.

L. B. Huang and R. Lim. Identification of injury-induced mitotic cells in adult rat cerebral cortex by neuron-specific markers. *Brain Res.Dev.Brain Res.* 51 (1):123-127, 1990.

A. B. Huber, A. L. Kolodkin, D. D. Ginty, and J. F. Cloutier. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annu.Rev.Neurosci.* 26:509-563, 2003.

J. J. Huerta, R. Diaz-Trelles, F. J. Naves, M. M. Llamosas, M. E. Del Valle, and J. A. Vega. Epidermal growth factor receptor in adult human dorsal root ganglia. *Anat.Embryol.(Berl)* 194 (3):253-257, 1996.

K. Husmann, A. Faissner, and M. Schachner. Tenascin promotes cerebellar granule cell migration and neurite outgrowth by different domains in the fibronectin type III repeats. *J.Cell Biol.* 116 (6):1475-1486, 1992.

D. Ilic, Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377 (6549):539-544, 1995.

K. Imai, M. Kusakabe, T. Sakakura, I. Nakanishi, and Y. Okada. Susceptibility of tenascin to degradation by matrix metalloproteinases and serine proteinases. *FEBS Lett.* 352 (2):216-218, 1994.

S. Inagaki, T. Furuyama, and Y. Iwahashi. Identification of a member of mouse semaphorin family. *FEBS Lett.* 370 (3):269-272, 1995.

A. Irintchev, M. Zeschnigk, A. Starzinski-Powitz, and A. Wernig . Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev.Dyn.* 199 (4):326-337, 1994.

A. Irintchev, J. D. Rosenblatt, M. J. Cullen, M. Zweyer, and A. Wernig. Ectopic skeletal muscles derived from myoblasts implanted under the skin. *J.Cell Sci.* 111 (Pt 22):3287-3297, 1998.

A. Irintchev, A. Rollenhagen, E. Troncoso, J. Z. Kiss, and M. Schachner. Structural and functional aberrations in the cerebral cortex of tenascin-C deficient mice. *Cereb.Cortex* 15 (7):950-962, 2005.

T. S. Jacques, J. B. Relvas, S. Nishimura, R. Pytela, G. M. Edwards, C. H. Streuli, and C. ffrench-Constant. Neural precursor cell chain migration and division are regulated through different beta1 integrins. *Development* 125 (16):3167-3177, 1998.

Y. Jinno, G. T. Merlino, and I. Pastan. A novel effect of EGF on mRNA stability. *Nucleic Acids Res.* 16 (11):4957-4966, 1988.

A. Joester and A. Faissner. Evidence for combinatorial variability of tenascin-C isoforms and developmental regulation in the mouse central nervous system. *J.Biol.Chem.* 274 (24):17144-17151, 1999.

A. Joester and A. Faissner. The structure and function of tenascins in the nervous system. *Matrix Biol.* 20 (1):13-22, 2001.

C. B. Johansson, M. Svensson, L. Wallstedt, A. M. Janson, and J. Frisen. Neural stem cells in the adult human brain. *Exp.Cell Res.* 253 (2):733-736, 1999.

K. K. Johe, T. G. Hazel, T. Muller, M. M. Dugich-Djordjevic, and R. D. McKay. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* 10 (24):3129-3140, 1996.

F. S. Jones, S. Hoffman, B. A. Cunningham, and G. M. Edelman. A detailed structural model of cytotactin: protein homologies, alternative RNA splicing, and binding regions. *Proc.Natl.Acad.Sci.U.S.A* 86 (6):1905-1909, 1989.

F. S. Jones and P. L. Jones. The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Dev.Dyn.* 218 (2):235-259, 2000.

P. L. Jones, J. Crack, and M. Rabinovitch. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J.Cell Biol.* 139 (1):279-293, 1997.

E. A. Joosten and A. A. Gribnau. Immunocytochemical localization of cell adhesion molecule L1 in developing rat pyramidal tract. *Neurosci.Lett.* 100 (1-3):94-98, 1989.

M. Jung, P. Pesheva, M. Schachner, and J. Trotter. Astrocytes and neurons regulate the expression of the neural recognition molecule janusin by cultured oligodendrocytes. *Glia* 9 (3):163-175, 1993.

G. Kadmon, M. Eckert, M. Sammar, M. Schachner, and P. Altevogt. Nectadrin, the heat-stable antigen, is a cell adhesion molecule. *J.Cell Biol.* 118 (5):1245-1258, 1992.

G. Kadmon, Halbach F. Bohlen und, M. Schachner, and P. Altevogt. Differential, LFA-1-sensitive effects of antibodies to nectadrin, the heat-stable antigen, on B lymphoblast aggregation and signal transduction. *Biochem.Biophys.Res.Commun.* 198 (3):1209-1215, 1994.

G. Kadmon, Halbach F. Bohlen und, R. Horstkorte, M. Eckert, P. Altevogt, and M. Schachner. Evidence for cis interaction and cooperative signalling by the heat-stable antigen nectadrin (murine CD24) and the cell adhesion molecule L1 in neurons. *Eur.J.Neurosci.* 7 (5):993-1004, 1995.

A. Kalyani, K. Hobson, and M. S. Rao. Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. *Dev.Biol.* 186 (2):202-223, 1997.

H. Kamiguchi, M. L. Hlavin, M. Yamasaki, and V. Lemmon. Adhesion molecules and inherited diseases of the human nervous system. *Annu.Rev.Neurosci.* 21:97-125, 1998.

J. P. Kapfhammer and J. A. Raper. Collapse of growth cone structure on contact with specific neurites in culture. *J.Neurosci.* 7 (1):201-212, 1987.

M. S. Kaplan and J. W. Hinds. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science* 197 (4308):1092-1094, 1977.

M. S. Kaplan. Neurogenesis in the 3-month-old rat visual cortex. *J.Comp Neurol.* 195 (2):323-338, 1981.

A. Kawakami, T. Kitsukawa, S. Takagi, and H. Fujisawa. Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. *J.Neurobiol.* 29 (1):1-17, 1996.

H. Kawano, K. Ohyama, K. Kawamura, and I. Nagatsu. Migration of dopaminergic neurons in the embryonic mesencephalon of mice. *Brain Res.Dev.Brain Res.* 86 (1-2):101-113, 1995.

T. Kawasaki, Y. Bekku, F. Suto, T. Kitsukawa, M. Taniguchi, I. Nagatsu, T. Nagatsu, K. Itoh, T. Yagi, and H. Fujisawa. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. *Development* 129 (3):671-680, 2002.

J. F. Kayyem, J. M. Roman, E. J. de la Rosa, U. Schwarz, and W. J. Dreyer. Bravo/Nr-CAM is closely related to the cell adhesion molecules L1 and Ng-CAM and has a similar heterodimer structure. *J.Cell Biol.* 118 (5):1259-1270, 1992.

T. E. Kennedy, T. Serafini, la Torre de, Jr., and M. Tessier-Lavigne. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 78 (3):425-435, 1994.

R. J. Keynes and C. D. Stern. Segmentation in the vertebrate nervous system. *Nature* 310 (5980):786-789, 1984.

R. J. Keynes and C. D. Stern. Mechanisms of vertebrate segmentation. *Development* 103 (3):413-429, 1988.

B. W. Kiernan, B. Gotz, A. Faissner, and C. ffrench-Constant. Tenascin-C inhibits oligodendrocyte precursor cell migration by both adhesion-dependent and adhesion-independent mechanisms. *Mol.Cell Neurosci.* 7 (4):322-335, 1996.

B. W. Kiernan, E. Garcion, J. Ferguson, E. E. Frost, E. M. Torres, S. B. Dunnett, Y. Saga, S. Aizawa, A. Faissner, R. Kaur, R. J. Franklin, and C. ffrench-Constant. Myelination and behaviour of tenascin-C null transgenic mice. *Eur.J.Neurosci.* 11 (9):3082-3092, 1999.

K. Kikuchi, H. Ishida, and T. Kimura. Molecular cloning of a novel member of semaphorin family genes, semaphorin Z. *Brain Res.Mol.Brain Res.* 51 (1-2):229-237, 1997.

T. J. Kilpatrick and P. F. Bartlett. Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. *J.Neurosci.* 15 (5 Pt 1):3653-3661, 1995.

T. J. Kilpatrick, L. J. Richards, and P. F. Bartlett. The regulation of neural precursor cells within the mammalian brain. *Mol.Cell Neurosci.* 6 (1):2-15, 1995.

T. Kitsukawa, A. Shimono, A. Kawakami, H. Kondoh, and H. Fujisawa. Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* 121 (12):4309-4318, 1995.

T. Kitsukawa, M. Shimizu, M. Sanbo, T. Hirata, M. Taniguchi, Y. Bekku, T. Yagi, and H. Fujisawa. Neuropilin-semaphorin III/D-mediated

chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* 19 (5):995-1005, 1997.

R. Kleene, H. Yang, M. Kutsche, and M. Schachner. The neural recognition molecule L1 is a sialic acid-binding lectin for CD24, which induces promotion and inhibition of neurite outgrowth. *J.Biol.Chem.* 276 (24):21656-21663, 2001.

P. Kleihues, F. Soylemezoglu, B. Schauble, B. W. Scheithauer, and P. C. Burger. Histopathology, classification, and grading of gliomas. *Glia* 15 (3):211-221, 1995.

A. L. Kolodkin, D. J. Matthes, T. P. O'Connor, N. H. Patel, A. Admon, D. Bentley, and C. S. Goodman. Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron* 9 (5):831-845, 1992.

A. L. Kolodkin, D. J. Matthes, and C. S. Goodman. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* 75 (7):1389-1399, 1993.

A. L. Kolodkin, D. V. Levengood, E. G. Rowe, Y. T. Tai, R. J. Giger, and D. D. Ginty. Neuropilin is a semaphorin III receptor. *Cell* 90 (4):753-762, 1997.

H. I. Kornblum, R. J. Hussain, J. M. Bronstein, C. M. Gall, D. C. Lee, and K. B. Seroogy. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J.Comp Neurol.* 380 (2):243-261, 1997.

C. E. Krull, D. B. Morton, A. Faissner, M. Schachner, and L. P. Tolbert. Spatiotemporal pattern of expression of tenascin-like molecules in a developing insect olfactory system. *J.Neurobiol.* 25 (5):515-534, 1994.

J. Kruse, G. Keilhauer, A. Faissner, R. Timpl, and M. Schachner. The J1 glycoprotein--a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature* 316 (6024):146-148, 1985.

S. Kuchler, S. Lehmann, G. Vincendon, and J. P. Zanetta. Endogenous lectin cerebellar soluble lectin involved in myelination is absent from nonmyelinating Schwann cells. *J.Neurochem.* 58 (5):1768-1772, 1992.

U. K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 (5259):680-685, 1970.

B. Langenfeld-Oster, A. Faissner, A. Irintchev, and A. Wernig. Polyclonal antibodies against NCAM and tenascin delay endplate reinnervation. *J.Neurocytol.* 23 (10):591-604, 1994.

E. D. Laywell, U. Dorries, U. Bartsch, A. Faissner, M. Schachner, and D. A. Steindler. Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury. *Proc.Natl.Acad.Sci.U.S.A* 89 (7):2634-2638, 1992.

S. Lehmann, S. Kuchler, M. Theveniau, G. Vincendon, and J. P. Zanetta. An endogenous lectin and one of its neuronal glycoprotein ligands are involved in contact guidance of neuron migration. *Proc.Natl.Acad.Sci.U.S.A* 87 (16):6455-6459, 1990.

C. Lethias, Y. Descollonges, M. M. Boutillon, and R. Garrone. Flexilin: a new extracellular matrix glycoprotein localized on collagen fibrils. *Matrix Biol.* 15 (1):11-19, 1996.

H. Li, D. K. Wu, and S. L. Sullivan. Characterization and expression of sema4g, a novel member of the semaphorin gene family. *Mech.Dev.* 87 (1-2):169-173, 1999.

H. Liao, W. Y. Bu, T. H. Wang, S. Ahmed, and Z. C. Xiao. Tenascin-R plays a role in neuroprotection via its distinct domains that coordinate to modulate the microglia function. *J.Biol.Chem.* 280 (9):8316-8323, 2005.

L. Lillien. Changes in retinal cell fate induced by overexpression of EGF receptor. *Nature* 377 (6545):158-162, 1995.

L. Lillien and H. Raphael. BMP and FGF regulate the development of EGF-responsive neural progenitor cells. *Development* 127 (22):4993-5005, 2000.

A. Lochter, L. Vaughan, A. Kaplony, A. Prochiantz, M. Schachner, and A. Faissner. J1/tenascin in substrate-bound and soluble form displays contrary effects on neurite outgrowth. *J.Cell Biol.* 113 (5):1159-1171, 1991.

A. Lochter and M. Schachner. Tenascin and extracellular matrix glycoproteins: from promotion to polarization of neurite growth in vitro. *J.Neurosci.* 13 (9):3986-4000, 1993.

A. Lochter, J. Taylor, B. Fuss, and M. Schachner. The extracellular matrix molecule janusin regulates neuronal morphology in a subst. *Eur.J.Neurosci.* 6 (4):597-606, 1994.

M. Loeffler, T. Bratke, U. Paulus, Y. Q. Li, and C. S. Potten. Clonality and life cycles of intestinal crypts explained by a state dependent stochastic model of epithelial stem cell organization. *J.Theor.Biol.* 186 (1):41-54, 1997.

C. Lois and A. Alvarez-Buylla. Long-distance neuronal migration in the adult mammalian brain. *Science* 264 (5162):1145-1148, 1994.

Y. Luo, D. Raible, and J. A. Raper. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 75 (2):217-227, 1993.

M. B. Luskin. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11 (1):173-189, 1993.

P. C. Mabie, M. F. Mehler, and J. A. Kessler. Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype. *J.Neurosci.* 19 (16):7077-7088, 1999.

E. J. Mackie, I. Thesleff, and R. Chiquet-Ehrismann. Tenascin is associated with chondrogenic and osteogenic differentiation in vivo and promotes chondrogenesis in vitro. *J.Cell Biol.* 105 (6 Pt 1):2569-2579, 1987.

E. J. Mackie, W. Halfter, and D. Liverani. Induction of tenascin in healing wounds. *J.Cell Biol.* 107 (6 Pt 2):2757-2767, 1988.

A. J. MacLennan, D. L. McLaurin, L. Marks, E. N. Vinson, M. Pfeifer, S. V. Szulc, M. B. Heaton, and N. Lee. Immunohistochemical localization of netrin-1 in the embryonic chick nervous system. *J.Neurosci.* 17 (14):5466-5479, 1997.

O. Marin and J. L. Rubenstein. Cell migration in the forebrain. *Annu.Rev.Neurosci.* 26:441-483, 2003.

M. D. Mark, M. Lohrum, and A. W. Puschel. Patterning neuronal connections by chemorepulsion: the semaphorins. *Cell Tissue Res.* 290 (2):299-306, 1997.

G. R. Martin. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.

Proc.Natl.Acad.Sci.U.S.A 78 (12):7634-7638, 1981.

R. Martini. Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. *J.Neurocytol.* 23 (1):1-28, 1994.

D. J. Matthes, H. Sink, A. L. Kolodkin, and C. S. Goodman. Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell* 81 (4):631-639, 1995.

J. W. McDonald, X. Z. Liu, Y. Qu, S. Liu, S. K. Mickey, D. Turetsky, D. I. Gottlieb, and D. W. Choi. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat.Med.* 5 (12):1410-1412, 1999.

A. P. McMahon and A. Bradley. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62 (6):1073-1085, 1990.

R. M. Mege, M. Nicolet, M. Pincon-Raymond, M. Murawsky, and F. Rieger. Cytotactin is involved in synaptogenesis during regeneration of the frog neuromuscular system. *Dev.Biol.* 149 (2):381-394, 1992.

S. Meiners and H. M. Geller. Long and Short Splice Variants of Human Tenascin Differentially Regulate Neurite Outgrowth. *Mol.Cell Neurosci.* 10 (1/2):100-116, 1997.

S. Meiners, M. L. Mercado, M. S. Kamal, and H. M. Geller. Tenascin-C contains domains that independently regulate neurite outgrowth and neurite guidance. *J.Neurosci.* 19 (19):8443-8453, 1999.

S. Meiners, M. S. Kamal, and M. L. Mercado. Identification of a neurite outgrowth-promoting motif within the alternatively spliced region of human tenascin-C. *J.Neurosci.* 21 (18):7215-7225, 2001.

M. L. Mercado, A. Kamal, H. Y. Liu, S. R. Gross, R. Movahed, and S. Meiners. Neurite outgrowth by the alternatively spliced region of human tenascin-C is mediated by neuronal alpha7beta1 integrin. *J.Neurosci.* 24 (1):238-247, 2004.

E. K. Messersmith, E. D. Leonardo, C. J. Shatz, M. Tessier-Lavigne , C. S. Goodman, and A. L. Kolodkin. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* 14 (5):949-959, 1995.

P. Milev, D. Fischer, M. Haring, T. Schulthess, R. K. Margolis, R. Chiquet-Ehrismann, and R. U. Margolis. The fibrinogen-like globe of tenascin-C mediates its interactions with neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta. *J.Biol.Chem.* 272 (24):15501-15509, 1997.

P. Milev, A. Chiba, M. Haring, H. Rauvala, M. Schachner, B. Ranscht, R. K. Margolis, and R. U. Margolis. High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta with tenascin-R, amphoterin, and the heparin-binding growth-associated molecule. *J.Biol.Chem.* 273 (12):6998-7005, 1998.

R. Milner, E. Frost, S. Nishimura, M. Delcommenne, C. Streuli, R. Pytela, and C. ffrench-Constant. Expression of alpha vbeta3 and alpha vbeta8 integrins during oligodendrocyte precursor differentiation in the presence and absence of axons. *Glia* 21 (4):350-360, 1997.

G. L. Ming, H. J. Song, B. Berninger, C. E. Holt, M. Tessier-Lavigne, and M. M. Poo. cAMP-

dependent growth cone guidance by netrin-1. *Neuron* 19 (6):1225-1235, 1997.

K. Mirnics and H. R. Koerber. Prenatal development of rat primary afferent fibers: II. Central projections. *J.Comp Neurol.* 355 (4):601-614, 1995.

N. Mitrovic, U. Dorries, and M. Schachner. Expression of the extracellular matrix glycoprotein tenascin in the somatosensory cortex of the mouse during postnatal development: an immunocytochemical and in situ hybridization analysis. *J.Neurocytol.* 23 (6):364-378, 1994.

N. Mitrovic and M. Schachner. Detection of tenascin-C in the nervous system of the tenascin-C mutant mouse. *J.Neurosci.Res.* 42 (5):710-717, 1995.

M. Montag-Sallaz and D. Montag. Severe cognitive and motor coordination deficits in tenascin-R-deficient mice. *Genes Brain Behav.* 2 (1):20-31, 2003.

C. Moreau-Fauvarque, A. Kumanogoh, E. Camand, C. Jaillard, G. Barbin, I. Boquet, C. Love, E. Y. Jones, H. Kikutani, C. Lubetzki, I. Dusart, and A. Chedotal. The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is expressed on oligodendrocytes and upregulated after CNS lesion. *J.Neurosci.* 23 (27):9229-9239, 2003.

Y. Morel, J. Bristow, S. E. Gitelman, and W. L. Miller. Transcript encoded on the opposite strand of the human steroid 21-hydroxylase/complement component C4 gene locus. *Proc.Natl.Acad.Sci.U.S.A* 86 (17):6582-6586, 1989.

S. J. Morrison, D. E. Wright, S. H. Cheshier, and I. L. Weissman. Hematopoietic stem cells: challenges to expectations. *Curr.Opin.Immunol.* 9 (2):216-221, 1997.

B. K. Mueller. Growth cone guidance: first steps towards a deeper understanding. *Annu.Rev.Neurosci.* 22:351-388, 1999.

R. J. Mullen, C. R. Buck, and A. M. Smith. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116 (1):201-211, 1992.

J. E. Murphy-Ullrich, V. A. Lightner, I. Aukhil, Y. Z. Yan, H. P. Erickson, and M. Hook. Focal adhesion integrity is downregulated by the alternatively spliced domain of human tenascin. *J.Cell Biol.* 115 (4):1127-1136, 1991.

M. Murray, D. Kim, Y. Liu, C. Tobias, A. Tessler, and I. Fischer. Transplantation of genetically modified cells contributes to repair and recovery from spinal injury. *Brain Res.Brain Res.Rev.* 40 (1-3):292-300, 2002.

M. Nakic, N. Mitrovic, G. Sperk, and M. Schachner. Kainic acid activates transient expression of tenascin-C in the adult rat hippocampus. *J.Neurosci.Res.* 44 (4):355-362, 1996.

M. Nakic, D. Manahan-Vaughan, K. G. Reymann, and M. Schachner. Long-term potentiation in vivo increases rat hippocampal tenascin-C expression. *J.Neurobiol.* 37 (3):393-404, 1998.

J. Nedelec, M. Pierres, H. Moreau, J. Barbet, P. Naquet, C. Faivre-Sarrailh, and G. Rougon. Isolation and characterization of a novel glycosyl-phosphatidylinositol-anchored glycoconjugate expressed by developing neurons. *Eur.J.Biochem.* 203 (3):433-442, 1992.

J. Neidhardt, S. Fehr, M. Kutsche, J. Lohler, and M. Schachner. Tenascin-N: characterization of a

novel member of the tenascin family that mediates neurite repulsion from hippocampal explants. *Mol.Cell Neurosci.* 23 (2):193-209, 2003.

P. J. Nielsen, B. Lorenz, A. M. Muller, R. H. Wenger, F. Brombacher, M. Simon, Weid T. von der, W. J. Langhorne, H. Mossmann, and G. Kohler. Altered erythrocytes and a leaky block in B-cell development in CD24/HSA-deficient mice. *Blood* 89 (3):1058-1067, 1997.

V. Nieoullon, R. Belvindrah, G. Rougon, and G. Chazal. mCD24 regulates proliferation of neuronal committed precursors in the subventricular zone. *Mol.Cell Neurosci.* 28 (3):462-474, 2005.

A. Nikonenko, S. Schmidt, G. Skibo, G. Bruckner, and M. Schachner. Tenascin-R-deficient mice show structural alterations of symmetric perisomatic synapses in the CA1 region of the hippocampus. *J.Comp Neurol.* 456 (4):338-349, 2003.

T. Nishio, S. Kawaguchi, T. Iseda, T. Kawasaki, and T. Hase. Secretion of tenascin-C by cultured astrocytes: regulation of cell proliferation and process elongation. *Brain Res.* 990 (1-2):129-140, 2003.

U. Norenberg, H. Wille, J. M. Wolff, R. Frank, and F. G. Rathjen. The chicken neural extracellular matrix molecule restrictin: similarity with EGF-, fibronectin type III-, and fibrinogen-like motifs. *Neuron* 8 (5):849-863, 1992.

U. Norenberg, M. Hubert, T. Brummendorf, A. Tarnok, and F. G. Rathjen. Characterization of functional domains of the tenascin-R (restrictin) polypeptide: cell attachment site, binding with F11, and enhancement of F11-mediated neurite outgrowth by tenascin-R. *J.Cell Biol.* 130 (2):473-484, 1995.

M. Okabe, M. Ikawa, K. Kominami, T. Nakanishi, and Y. Nishimune. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* 407 (3):313-319, 1997.

M. Olsson, K. Campbell, and D. H. Turnbull. Specification of mouse telencephalic and mid-hindbrain progenitors following heterotopic ultrasound-guided embryonic transplantation. *Neuron* 19 (4):761-772, 1997.

J. Ourednik, V. Ourednik, M. Bastmeyer, and M. Schachner. Ectopic expression of the neural cell adhesion molecule L1 in astrocytes leads to changes in the development of the corticospinal tract. *Eur.J.Neurosci.* 14 (9):1464-1474, 2001.

S. Ozaki and W. D. Snider. Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. *J.Comp Neurol.* 380 (2):215-229, 1997.

L. Patthy. Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. *Cell* 61 (1):13-14, 1990.

P. Pesheva, E. Spiess, and M. Schachner. J1-160 and J1-180 are oligodendrocyte-secreted nonpermissive substrates for cell adhesion. *J.Cell Biol.* 109 (4 Pt 1):1765-1778, 1989.

P. Pesheva, R. Probstmeier, E. Spiess, and M. Schachner. Divalent Cations Modulate the Inhibitory Substrate Properties of Murine Glia-derived J1-160 and J1-180 Extracellular Matrix Glycoproteins for Neuronal Adhesion. *Eur.J.Neurosci.* 3 (4):356-365, 1991.

P. Pesheva, G. Gennarini, C. Goridis, and M. Schachner. The F3/11 cell adhesion molecule mediates the repulsion of neurons by the extracellular matrix glycoprotein J1-160/180. *Neuron* 10 (1):69-82, 1993.

P. Pesheva, R. Probstmeier, A. P. Skubitz, J. B. McCarthy, L. T. Furcht, and M. Schachner. Tenascin-R (J1 160/180 inhibits fibronectin-mediated cell adhesion--functional relatedness to tenascin-C. *J.Cell Sci.* 107 (Pt 8):2323-2333, 1994.

P. Pesheva, S. Gloor, M. Schachner, and R. Probstmeier. Tenascin-R is an intrinsic autocrine factor for oligodendrocyte differentiation and promotes cell adhesion by a sulfatide-mediated mechanism. *J.Neurosci.* 17 (12):4642-4651, 1997.

P. Pesheva and R. Probstmeier. The yin and yang of tenascin-R in CNS development and pathology. *Prog.Neurobiol.* 61 (5):465-493, 2000.

G. R. Phillips, L. A. Krushel, and K. L. Crossin. Domains of tenascin involved in glioma migration. *J.Cell Sci.* 111 (Pt 8):1095-1104, 1998.

R. R. Pindzola, C. Doller, and J. Silver. Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Dev.Biol.* 156 (1):34-48, 1993.

S. Pluchino, A. Quattrini, E. Brambilla, A. Gritti, G. Salani, G. Dina, R. Galli, U. Del Carro, S. Amadio, A. Bergami, R. Furlan, G. Comi, A. L. Vescovi, and G. Martino. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422 (6933):688-694, 2003.

F. Polleux, T. Morrow, and A. Ghosh. Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* 404 (6778):567-573, 2000.

N. Pringle, E. J. Collarini, M. J. Mosley, C. H. Heldin, B. Westermark, and W. D. Richardson. PDGF A chain homodimers drive proliferation of bipotential (O-2A) glial progenitor cells in the developing rat optic nerve. *EMBO J.* 8 (4):1049-1056, 1989.

R. Probstmeier, M. Michels, T. Franz, B. M. Chan, and P. Pesheva. Tenascin-R interferes with integrin-dependent oligodendrocyte precursor cell adhesion by a ganglioside-mediated signalling mechanism. *Eur.J.Neurosci.* 11 (7):2474-2488, 1999.

R. Probstmeier, C. C. Stichel, H. W. Muller, H. Asou, and P. Pesheva. Chondroitin sulfates expressed on oligodendrocyte-derived tenascin-R are involved in neural cell recognition. Functional implications during CNS development and regeneration. *J.Neurosci.Res.* 60 (1):21-36, 2000.

R. Probstmeier, J. Nellen, S. Gloor, A. Wernig, and P. Pesheva. Tenascin-R is expressed by Schwann cells in the peripheral nervous system. *J.Neurosci.Res.* 64 (1):70-78, 2001.

A. W. Puschel, R. H. Adams, and H. Betz. Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. *Neuron* 14 (5):941-948, 1995.

A. W. Puschel, R. H. Adams, and H. Betz. The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. *Mol.Cell Neurosci.* 7 (5):419-431, 1996.

X. Qian, A. A. Davis, S. K. Goderie, and S. Temple. FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. *Neuron* 18 (1):81-93, 1997.

J. Qiu, D. Cai, and M. T. Filbin. Glial inhibition of nerve regeneration in the mature mammalian CNS. *Glia* 29 (2):166-174, 2000.

X. Qu, H. Wei, Y. Zhai, H. Que, Q. Chen, F. Tang, Y. Wu, G. Xing, Y. Zhu, S. Liu, M. Fan, and F. He. Identification, characterization, and functional study of the two novel human members of the semaphorin gene family. *J.Biol.Chem.* 277 (38):35574-35585, 2002.

S. M. Quinn, W. M. Walters, A. L. Vescovi, and S. R. Whittemore . Lineage restriction of neuroepithelial precursor cells from fetal human spinal cord. *J.Neurosci.Res.* 57 (5):590-602, 1999.

S. A. Rabacchi, J. M. Solowska, B. Kruk, Y. Luo, J. A. Raper, and D. H. Baird. Collapsin-1/semaphorin-III/D is regulated developmentally in Purkinje cells and collapses pontocerebellar mossy fiber neuronal growth cones. *J.Neurosci.* 19 (11):4437-4448, 1999.

O. Raineteau, L. Rietschin, G. Gradwohl, F. Guillemot, and B. H. Gahwiler. Neurogenesis in hippocampal slice cultures. *Mol.Cell Neurosci.* 26 (2):241-250, 2004.

J. A. Raper and J. P. Kapfhammer. The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. *Neuron* 4 (1):21-29, 1990.

F. G. Rathjen and M. Schachner. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO J.* 3 (1):1-10, 1984.

F. G. Rathjen, U. Norenberg, and H. Volkmer. Glycoproteins implicated in neural cell adhesion and axonal growth. *Biochem.Soc.Trans.* 20 (2):405-409, 1992.

B. S. Reinoso and A. J. Castro. A study of corticospinal remodelling using retrograde fluorescent tracers in rats. *Exp.Brain Res.* 74 (2):387-394, 1989.

X. D. Ren, W. B. Kiosses, D. J. Sieg, C. A. Otey, D. D. Schlaepfer, and M. A. Schwartz. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J.Cell Sci.* 113 (Pt 20):3673-3678, 2000.

W. J. Rettig, T. J. Triche, and P. Garin-Chesa. Stimulation of human neuronectin secretion by brain-derived growth factors. *Brain Res.* 487 (1):171-177, 1989.

B. A. Reynolds and S. Weiss. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255 (5052):1707-1710, 1992.

B. A. Reynolds and S. Weiss. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev.Biol.* 175 (1):1-13, 1996.

I. Richard, M. Ader, V. Sytnyk, A. Dityatev, G. Richard, M. Schachner, and U. Bartsch. Electroporation-based gene transfer for efficient transfection of neural precursor cells. *Brain Res.Mol.Brain Res.* 138 (2):182-190, 2005.

R. M. Richardson, W. C. Broaddus, K. L. Holloway, and H. L. Fillmore. Grafts of adult subependymal zone neuronal progenitor cells rescue hemiparkinsonian behavioral decline. *Brain Res.* 1032 (1-2):11-22, 2005.

F. Rigato, J. Garwood, V. Calco, N. Heck, C. Faivre-Sarrailh, and A. Faissner. Tenascin-C promotes neurite outgrowth of embryonic hippocampal neurons through the alternatively spliced fibronectin type III BD domains via activation of the cell adhesion molecule F3/contactin. *J.Neurosci.* 22 (15):6596-6609, 2002.

B. Rohm, B. Rahim, B. Kleiber, I. Hovatta, and A. W. Puschel. The semaphorin 3A receptor may

directly regulate the activity of small GTPases. *FEBS Lett.* 486 (1):68-72, 2000.

B. Rolf, M. Bastmeyer, M. Schachner, and U. Bartsch. Pathfinding errors of corticospinal axons in neural cell adhesion molecule-deficient mice. *J.Neurosci.* 22 (19):8357-8362, 2002.

Y. Saga, T. Tsukamoto, N. Jing, M. Kusakabe, and T. Sakakura. Murine tenascin: cDNA cloning, structure and temporal expression of isoforms. *Gene* 104 (2):177-185, 1991.

A. Saghatelyan, A. de Chevigny, M. Schachner, and P. M. Lledo. Tenascin-R mediates activity-dependent recruitment of neuroblasts in the adult mouse forebrain. *Nat.Neurosci.* 7 (4):347-356, 2004.

A. K. Saghatelyan, S. Gorissen, M. Albert, B. Hertlein, M. Schachner, and A. Dityatev. The extracellular matrix molecule tenascin-R and its HNK-1 carbohydrate modulate perisomatic inhibition and long-term potentiation in the CA1 region of the hippocampus. *Eur.J.Neurosci.* 12 (9):3331-3342, 2000.

A. K. Saghatelyan, A. Dityatev, S. Schmidt, T. Schuster, U. Bartsch, and M. Schachner. Reduced perisomatic inhibition, increased excitatory transmission, and impaired long-term potentiation in mice deficient for the extracellular matrix glycoprotein tenascin-R. *Mol.Cell Neurosci.* 17 (1):226-240, 2001.

M. Salmivirta, K. Elenius, S. Vainio, U. Hofer, R. Chiquet-Ehrismann, I. Thesleff, and M. Jalkanen. Syndecan from embryonic tooth mesenchyme binds tenascin. *J.Biol.Chem.* 266 (12):7733-7739, 1991.

S. R. Salton, C. Richter-Landsberg , L. A. Greene, and M. L. Shelanski. Nerve growth factor-inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. *J.Neurosci.* 3 (3):441-454, 1983.

J Sambrook, E. F. Fritch, and T. Maniatis. Molecular Cloning: A Laboratory Manual. Anonymous. Anonymous. Cold Spring Harbor: *Cold Spring Harbor Laboratory* , 1989.

M. Sawada, N. Kondo, and T. Marunouchi. Programmed cell death of PC12 induced by adenovirus E1A. *Neurosci.Lett.* 191 (3):173-176, 1995.

S. Schumacher and E. M. Stube. Regulated binding of the fibrinogen-like domains of tenascin-R and tenascin-C to the neural EGF family member CALEB. *J.Neurochem.* 87 (5):1213-1223, 2003.

M. Seiki. The cell surface: the stage for matrix metalloproteinase regulation of migration. *Curr.Opin.Cell Biol.* 14 (5):624-632, 2002.

T. Serafini, T. E. Kennedy, M. J. Galko, C. Mirzayan, T. M. Jessell, and M. Tessier-Lavigne. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* 78 (3):409-424, 1994.

T. Serafini, S. A. Colamarino, E. D. Leonardo, H. Wang, R. Beddington, W. C. Skarnes, and M. Tessier-Lavigne. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87 (6):1001-1014, 1996.

D. Shewan, V. Calaora, P. Nielsen, J. Cohen, G. Rougon, and H. Moreau. mCD24, a glycoprotein transiently expressed by neurons, is an inhibitor of neurite outgrowth. *J.Neurosci.* 16 (8):2624-2634, 1996.

L. S. Shihabuddin, P. J. Horner, J. Ray, and F. H. Gage. Adult spinal cord stem cells generate neurons

after transplantation in the adult dentate gyrus. *J.Neurosci.* 20 (23):8727-8735, 2000.

D. T. Shima and C. Mailhos. Vascular developmental biology: getting nervous. *Curr.Opin.Genet.Dev.* 10 (5):536-542, 2000.

T. Shirasawa, T. Akashi, K. Sakamoto, H. Takahashi, N. Maruyama, and K. Hirokawa. Gene expression of CD24 core peptide molecule in developing brain and developing non-neural tissues. *Dev.Dyn.* 198 (1):1-13, 1993.

A. Siri, B. Carnemolla, M. Saginati, A. Leprini, G. Casari, F. Baralle, and L. Zardi. Human tenascin: primary structure, pre-mRNA splicing patterns and localization of the epitopes recognized by two monoclonal antibodies. *Nucleic Acids Res.* 19 (3):525-531, 1991.

E. Y. Snyder, C. Yoon, J. D. Flax, and J. D. Macklis. Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. *Proc.Natl.Acad.Sci.U.S.A* 94 (21):11663-11668, 1997.

M. V. Sofroniew and U. Schrell. Long-term storage and regular repeated use of diluted antisera in glass staining jars for increased sensitivity, reproducibility, and convenience of single- and two-color light microscopic immunocytochemistry. *J.Histochem.Cytochem.* 30 (6):504-511, 1982.

H. Song, G. Ming, Z. He, M. Lehmann, L. McKerracher, M. Tessier-Lavigne, and M. Poo. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* 281 (5382):1515-1518, 1998.

H. J. Song, G. L. Ming, and M. M. Poo. cAMP-induced switching in turning direction of nerve growth cones. *Nature* 388 (6639):275-279, 1997.

A. Spradling, D. Drummond-Barbosa, and T. Kai. Stem cells find their niche. *Nature* 414 (6859):98-104, 2001.

T. Springer, G. Galfre, D. S. Secher, and C. Milstein. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur.J.Immunol.* 8 (8):539-551, 1978.

J. Srinivasan, M. Schachner, and W. A. Catterall. Interaction of voltage-gated sodium channels with the extracellular matrix molecules tenascin-C and tenascin-R. *Proc.Natl.Acad.Sci.U.S.A* 95 (26):15753-15757, 1998.

P. Sriramarao and M. A. Bourdon. A novel tenascin type III repeat is part of a complex of tenascin mRNA alternative splices. *Nucleic Acids Res.* 21 (1):163-168, 1993.

D. A. Steindler, N. G. Cooper, A. Faissner, and M. Schachner. Boundaries defined by adhesion molecules during development of the cerebral cortex: the J1/tenascin glycoprotein in the mouse somatosensory cortical barrel field. *Dev.Biol.* 131 (1):243-260, 1989.

D. A. Steindler, D. Settles, H. P. Erickson, E. D. Laywell, A. Yoshiki, A. Faissner, and M. Kusakabe. Tenascin knockout mice: barrels, boundary molecules, and glial scars. *J.Neurosci.* 15 (3 Pt 1):1971-1983, 1995.

L. Stoppini, P. A. Buchs, and D. Muller. A simple method for organotypic cultures of nervous tissue. *J.Neurosci.Methods* 37 (2):173-182, 1991.

T. Strekalova, M. Sun, M. Sibbe, M. Evers, A. Dityatev, P. Gass, and M. Schachner. Fibronectin domains of extracellular matrix molecule tenascin-C modulate hippocampal learning and synaptic plasticity. *Mol.Cell Neurosci.* 21 (1):173-187, 2002.

J. O. Suhonen, D. A. Peterson, J. Ray, and F. H. Gage. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* 383 (6601):624-627, 1996.

H. Super, E. Soriano, and H. B. Uylings. The functions of the preplate in development and evolution of the neocortex and hippocampus. *Brain Res.Brain Res.Rev.* 27 (1):40-64, 1998.

O. N. Suslov, V. G. Kukekov, T. N. Ignatova, and D. A. Steindler . Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proc.Natl.Acad.Sci.U.S.A* 99 (22):14506-14511, 2002.

C. S. Swindle, K. T. Tran, T. D. Johnson, P. Banerjee, A. M. Mayes, L. Griffith, and A. Wells. Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. *J.Cell Biol.* 154 (2):459-468, 2001.

T. Takahashi, A. Fournier, F. Nakamura, L. H. Wang, Y. Murakami, R. G. Kalb, H. Fujisawa, and S. M. Strittmatter. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* 99 (1):59-69, 1999.

L. Tamagnone, S. Artigiani, H. Chen, Z. He, G. I. Ming, H. Song, A. Chedotal, M. L. Winberg, C. S. Goodman, M. Poo, M. Tessier-Lavigne, and P. M. Comoglio. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* 99 (1):71-80, 1999.

N. Tamamaki, K. Nakamura, K. Okamoto, and T. Kaneko. Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex. *Neurosci.Res.* 41 (1):51-60, 2001.

S. S. Tan, K. L. Crossin, S. Hoffman, and G. M. Edelman. Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. *Proc.Natl.Acad.Sci.U.S.A* 84 (22):7977-7981, 1987.

M. Taniguchi, S. Yuasa, H. Fujisawa, I. Naruse, S. Saga, M. Mishina, and T. Yagi. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* 19 (3):519-530, 1997.

M. Taniguchi and T. Shimizu. Characterization of a novel member of murine semaphorin family. *Biochem.Biophys.Res.Commun.* 314 (1):242-248, 2004.

P. Taupin and F. H. Gage. Adult neurogenesis and neural stem cells of the central nervous system in mammals. *J.Neurosci.Res.* 69 (6):745-749, 2002.

J. Taylor, P. Pesheva, and M. Schachner. Influence of janusin and tenascin on growth cone behavior in vitro. *J.Neurosci.Res.* 35 (4):347-362, 1993.

S. Temple. The development of neural stem cells. *Nature* 414 (6859):112-117, 2001.

T. Terashima. Anatomy, development and lesion-induced plasticity of rodent corticospinal tract. *Neurosci.Res.* 22 (2):139-161, 1995.

M. Tessier-Lavigne and C. S. Goodman. The molecular biology of axon guidance. *Science* 274 (5290):1123-1133, 1996.

D. T. Theodosis, K. Pierre, M. A. Cadoret, M. Allard, A. Faissner, and D. A. Poulain. Expression of high levels of the extracellular matrix glycoprotein, tenascin-C, in the normal adult hypothalamoneurohypophysial system. *J.Comp Neurol.* 379 (3):386-398, 1997.

I. Thesleff, E. Mackie, S. Vainio, and R. Chiquet-Ehrismann. Changes in the distribution of tenascin during tooth development. *Development* 101 (2):289-296, 1987.

A. Togari, M. Mogi, M. Arai, S. Yamamoto, and Y. Koshihara. Expression of mRNA for axon guidance molecules, such as semaphorin-III, netrins and neurotrophins, in human osteoblasts and osteoclasts. *Brain Res.* 878 (1-2):204-209, 2000.

E. Tongiorgi, R. R. Bernhardt, and M. Schachner. Zebrafish neurons express two L1-related molecules during early axonogenesis. *J.Neurosci.Res.* 42 (4):547-561, 1995.

V. Tropepe, M. Sibilia, B. G. Ciruna, J. Rossant, E. F. Wagner, and Kooy D. van der. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev.Biol.* 208 (1):166-188, 1999.

R. P. Tucker and S. E. McKay. The expression of tenascin by neural crest cells and glia. *Development* 112 (4):1031-1039, 1991.

R. P. Tucker, C. Hagios, and R. Chiquet-Ehrismann. Tenascin-Y in the developing and adult avian nervous system. *Dev.Neurosci.* 21 (2):126-133, 1999.

R. P. Tucker. Abnormal neural crest cell migration after the in vivo knockdown of tenascin-C expression with morpholino antisense oligonucleotides. *Dev.Dyn.* 222 (1):115-119, 2001.

R. E. van Kesteren and G. E. Spencer. The role of neurotransmitters in neurite outgrowth and synapse formation. *Rev.Neurosci.* 14 (3):217-231, 2003.

A. Varela-Echavarria, A. Tucker, A. W. Puschel, and S. Guthrie. Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. *Neuron* 18 (2):193-207, 1997.

B. Varnum-Finney, K. Venstrom, U. Muller, R. Kypta, C. Backus, M. Chiquet, and L. F. Reichardt. The integrin receptor alpha 8 beta 1 mediates interactions of embryonic chick motor and sensory neurons with tenascin-C. *Neuron* 14 (6):1213-1222, 1995.

L. Vaughan, S. Huber, M. Chiquet, and K. H. Winterhalter. A major, six-armed glycoprotein from embryonic cartilage. *EMBO J.* 6 (2):349-353, 1987.

A. L. Vescovi, E. A. Parati, A. Gritti, P. Poulin, M. Ferrario, E. Wanke, P. Frolichsthal-Schoeller, L. Cova, M. Arcellana-Panlilio, A. Colombo, and R. Galli. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp.Neurol.* 156 (1):71-83, 1999.

H. Volkmer, B. Hassel, J. M. Wolff, R. Frank, and F. G. Rathjen. Structure of the axonal surface recognition molecule neurofascin and its relationship to a neural subgroup of the immunoglobulin superfamily. *J.Cell Biol.* 118 (1):149-161, 1992.

H. Volkmer, U. Zacharias, U. Norenberg, and F. G. Rathjen. Dissection of complex molecular interactions of neurofascin with axonin-1, F11, and tenascin-R, which promote attachment and neurite formation of tectal cells. *J.Cell Biol.* 142 (4):1083-1093, 1998.

G. Vollmer, H. Michna, M. R. Schneider, and R. Knuppen. Stromal expression of tenascin is inversely correlated to epithelial differentiation of hormone dependent tissues. *J.Steroid Biochem.Mol.Biol.* 48 (5-6):487-494, 1994.

K. Wallner, C. Li, P. K. Shah, K. J. Wu, S. M. Schwartz, and B. G. Sharifi. EGF-Like domain of tenascin-C is proapoptotic for cultured smooth muscle cells . *Arterioscler.Thromb.Vasc.Biol.* 24 (8):1416-1421, 2004.

L. H. Wang and S. M. Strittmatter. A family of rat CRMP genes is differentially expressed in the nervous system. *J.Neurosci.* 16 (19):6197-6207, 1996.

P. Weber, P. Ferber, R. Fischer, K. H. Winterhalter, and L. Vaughan. Binding of contactin/F11 to the fibronectin type III domains 5 and 6 of tenascin is inhibited by heparin. *FEBS Lett.* 389 (3):304-308, 1996.

P. Weber, D. Montag, M. Schachner, and R. R. Bernhardt. Zebrafish tenascin-W, a new member of the tenascin family. *J.Neurobiol.* 35 (1):1-16, 1998.

P. Weber, U. Bartsch, M. N. Rasband, R. Czaniera, Y. Lang, H. Bluethmann, R. U. Margolis, S. R. Levinson, P. Shrager, D. Montag, and M. Schachner. Mice deficient for tenascin-R display alterations of the extracellular matrix and decreased axonal conduction velocities in the CNS. *J.Neurosci.* 19 (11):4245-4262, 1999.

B. Wehrle-Haller and M. Chiquet. Dual function of tenascin: simultaneous promotion of neurite growth and inhibition of glial migration. *J.Cell Sci.* 106 (Pt 2):597-610, 1993.

B. Wehrle and M. Chiquet. Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth in vitro. *Development* 110 (2):401-415, 1990.

S. Weiss, C. Dunne, J. Hewson, C. Wohl, M. Wheatley, A. C. Peterson, and B. A. Reynolds. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J.Neurosci.* 16 (23):7599-7609, 1996.

A. Weller, S. Beck, and P. Ekblom. Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. *J.Cell Biol.* 112 (2):355-362, 1991.

R. H. Wenger, M. Kopf, L. Nitschke, M. C. Lamers, G. Kohler, and P. J. Nielsen. B-cell maturation in chimaeric mice deficient for the heat stable antigen (HSA/mouse CD24). *Transgenic Res.* 4 (3):173-183, 1995.

A. Wennersten, X. Meier, S. Holmin, L. Wahlberg, and T. Mathiesen. Proliferation, migration, and differentiation of human neural stem/progenitor cells after transplantation into a rat model of traumatic brain injury. *J.Neurosurg.* 100 (1):88-96, 2004.

F. A. White and O. Behar. The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. *Dev.Biol.* 225 (1):79-86, 2000.

C. Winkler, R. A. Fricker, M. A. Gates, M. Olsson, J. P. Hammang, M. K. Carpenter, and A. Bjorklund. Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain. *Mol.Cell Neurosci.* 11 (3):99-116, 1998.

E. S. Wintergerst, B. Fuss, and U. Bartsch. Localization of janusin mRNA in the central nervous system of the developing and adult mouse. *Eur.J.Neurosci.* 5 (4):299-310, 1993.

E. S. Wintergerst, D. M. Vogt Weisenhorn, F. G. Rathjen, B. M. Riederer, S. Lambert, and M. R. Celio. Temporal and spatial appearance of the membrane cytoskeleton and perineuronal nets in the

rat neocortex. *Neurosci.Lett.* 209 (3):173-176, 1996.

A. Woodworth, D. Fiete, and J. U. Baenziger. Spatial and temporal regulation of tenascin-R glycosylation in the cerebellum. *J.Biol.Chem.* 277 (52):50941-50947, 2002.

D. E. Wright, F. A. White, R. W. Gerfen, I. Silos-Santiago, and W. D. Snider. The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. *J.Comp Neurol.* 361 (2):321-333, 1995.

R. H. Xiang, C. H. Hensel, D. K. Garcia, H. C. Carlson, K. Kok, M. C. Daly, K. Kerbacher, Berg A. van den, P. Veldhuis, C. H. Buys, and S. L. Naylor. Isolation of the human semaphorin III/F gene (SEMA3F) at chromosome 3p21, a region deleted in lung cancer. *Genomics* 32 (1):39-48, 1996.

Z. C. Xiao, J. Taylor, D. Montag, G. Rougon, and M. Schachner. Distinct effects of recombinant tenascin-R domains in neuronal cell functions and identification of the domain interacting with the neuronal recognition molecule F3/11. *Eur.J.Neurosci.* 8 (4):766-782, 1996.

Z. C. Xiao, R. Hillenbrand, M. Schachner, S. Thermes, G. Rougon, and S. Gomez. Signaling events following the interaction of the neuronal adhesion molecule F3 with the N-terminal domain of tenascin-R. *J.Neurosci.Res.* 49 (6):698-709, 1997.

Z. C. Xiao, D. S. Ragsdale, J. D. Malhotra, L. N. Mattei, P. E. Braun, M. Schachner, and L. L. Isom. Tenascin-R is a functional modulator of sodium channel beta subunits. *J.Biol.Chem.* 274 (37):26511-26517, 1999.

H. Yang, Z. C. Xiao, B. Becker, R. Hillenbrand, G. Rougon, and M. Schachner. Role for myelin-associated glycoprotein as a functional tenascin-R receptor. *J.Neurosci.Res.* 55 (6):687-701, 1999.

S. Yuasa. Bergmann glial development in the mouse cerebellum as revealed by tenascin expression. *Anat.Embryol.(Berl)* 194 (3):223-234, 1996.

Y. Zhang, P. N. Anderson, G. Campbell, H. Mohajeri, M. Schachner, and A. R. Lieberman. Tenascin-C expression by neurons and glial cells in the rat spinal cord: changes during postnatal development and after dorsal root or sciatic nerve injury. *J.Neurocytol.* 24 (8):585-601, 1995.

Y. Zhang, J. K. Winterbottom, M. Schachner, A. R. Lieberman, and P. N. Anderson. Tenascin-C expression and axonal sprouting following injury to the spinal dorsal columns in the adult rat. *J.Neurosci.Res.* 49 (4):433-450, 1997.

L. Zhuo, B. Sun, C. L. Zhang, A. Fine, S. Y. Chiu, and A. Messing. Live astrocytes visualized by green fluorescent protein in transgenic mice. *Dev.Biol.* 187 (1):36-42, 1997.

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May you build a ladder to the stars and climb on every run,
May you always know the truth and see the light surrounding you,
May you always be courageous, stand upright and be strong,
May your heart always be joyful and may your song always be sung.

(B.Dylan, 1974)

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I, Martin Hammond, certify that this dissertation by Ms Mirjam Sibbe is written in correct and comprehensible English.

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